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**PHYSIOLOGY OF COLD ACCLIMATION AND DEACCLIMATION  
RESPONSES OF COOL-SEASON GRASSES: CARBON AND HORMONE  
METABOLISM**

A Thesis Presented

by

Xian Guan

Submitted to the Graduate School of the  
University of Massachusetts in partial fulfillment  
of the requirements for the degree of  
**MASTER OF SCIENCE**

September 2014

Plant and Soil Sciences

**PHYSIOLOGY OF COLD ACCLIMATION AND DEACCLIMATION  
RESPONSES OF COOL-SEASON GRASSES: CARBON AND HORMONE  
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A Thesis presented

by

Xian Guan

Approved as to style and content by:

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Michelle DaCosta, Chair

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J. Scott Ebdon, Member

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Wesley R. Autio, Director  
Stockbridge School of Agriculture

## **DEDICATION**

To my parents and my husband, whose love and support always drives me chasing my dreams.

## **ACKNOWLEDGEMENTS**

First and foremost, I would like to express my sincere thanks to my advisor, Dr. Michelle DaCosta, who has provided endless advice, guidance and help through my three years of study at the University of Massachusetts Amherst. I would also like to thank my graduate committee members, Dr. J. Scott Ebdon, and Dr. Geunhwa Jung for all the advice and suggestions for my M.S. thesis research.

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## **ABSTRACT**

### **PHYSIOLOGY OF COLD ACCLIMATION AND DEACCLIMATION RESPONSES OF COOL-SEASON GRASSES: CARBON AND HORMONE METABOLISM**

SEPTEMBER 2014

XIAN GUAN, B.S., BEIJING FORESTRY UNIVERSITY

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Winter injury of cool-season turfgrasses in northern climates is a significant issue, leading to losses in turf cover and subsequent increased inputs for recovery. Despite the different potential causes for winter injury, the overall level of plant freezing tolerance has been shown to account for a majority of the variation in winter survival of grasses. Freezing tolerance is achieved through cold acclimation, based on a series of physiological and biochemical changes that increase cell stability at freezing temperatures. Winter injury can result from insufficient cold acclimation, or rapid cold deacclimation triggered by temperature fluctuations or freeze-thaw cycles. Previous research has been mostly conducted to investigate the mechanisms associated with cold acclimation, limited research was applied to deacclimation resistance. In order to enhance winter survival, and reduce turf losses and recovery costs, a better understanding of the underlying factors associated with cold acclimation and deacclimation is necessary. Therefore, the objectives of this thesis research are to: (1) evaluate the carbon metabolism factors attributing to different freezing tolerance capacity associated with cold acclimation and deacclimation of annual bluegrass and creeping bentgrass; (2) investigate the differences in hormone regulation of annual bluegrass and creeping bentgrass during cold acclimation and

deacclimation; and (3) identify physiological changes in response to cold acclimation and deacclimation among perennial ryegrass genotypes contrasting in freezing tolerance. Overall, our research found that the fast up-regulation of carbon metabolism activities (chlorophyll fluorescence, photosynthesis, respiration) during deacclimation was associated with losses in freezing tolerance. In addition, changes in hormone content, such as abscisic acid, auxin, salicylic acid, and jasmonic acid, at both leaf and crown level, contributed to differences in deacclimation resistance. Lastly, increased crown moisture content during deacclimation was also found to be responsible for the losses in freezing tolerance. Although these factors may aid in a faster recovery in response to temperature increases during late winter and early spring, these physiological changes may also make the plants more susceptible to freezing injuries if plants are once again exposed to freezing temperatures.

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## CHAPTER 1

### LITERATURE REVIEW

Temperature is a significant factor influencing plant geographic distribution, quality, and productivity. Plants exposed to temperature extremes, including supra-optimal and sub-optimal temperatures, can result in significant stress and subsequent loss of vegetative cover. In temperate regions, winter injury has been cited as a major problem limiting the quality and production of various crop plants, including vegetables, fruits, cereals, turf and forage grasses (Chen et al., 1983; Saltveit and Morris, 1990; Lyons, 1973; Burke et al., 1976; Trenholm, 2000; Bélanger et al. 2002; Anderson et al., 2003). Although most research addressing issues with predicted increases in future global temperatures have focused on high temperature effects on crop plants in summer months, warming events that occur in the fall, winter, and/or spring may have significant impact on plant sensitivity to freezing damage (Cannell and Smith, 1986). Elevated temperatures may negatively impact survival by prolonging the fall growing season and prevent maximal cold hardening prior to freezing temperatures, while more frequent temperature fluctuations throughout winter months (freeze-thaw events) may result in premature losses in freezing tolerance (Bélanger et al., 2002; Thorsen and Höglind, 2010). As a result, it has become increasingly important to identify traits necessary for winter survival under both current and future winter climate scenarios.

Winter injury is a general term used to encompass damage to plants associated with one or multiples overwintering stresses, including desiccation, anoxia, crown hydration, low temperature fungi, and direct low temperature kill. In spite of the different potential causes for winter injury, the overall level of plant freezing tolerance has been shown to account for a majority of the variation in winter survival of plants,

including temperate grasses (Humphreys, 1989; Xiong and Fei, 2006; Hulke et al., 2008). Therefore, the focus of the following thesis will be to investigate physiological mechanisms contributing to differences in freezing tolerance among cool-season turfgrasses. The overall goal is to utilize this fundamental information to better inform management practices and turfgrass species/cultivar selection aimed at improving winter survival of turf stands.

### **Freezing Injury at the Cellular Level**

Damage to plants caused by low temperature can be generally categorized as two injuries, including chilling and freezing. Chilling injury refers to damage induced by low, non-freezing temperatures ( $> 0^{\circ}\text{C}$ ) and is primarily associated with cell membrane dysfunction (Lyons, 1973; Suzuki and Mittler, 2006; Reulland et al., 2009). Symptoms of chilling injury may include reduced growth rate, water-soaked tissues, as well as photooxidation of leaves. In general, tropical and subtropical plants are primarily susceptible to chilling injury (Taiz and Zeiger, 2006).

In contrast, freezing injury occurs when temperatures drop below freezing ( $< 0^{\circ}\text{C}$ ), and is attributed to both osmotic and oxidative stresses resulting from the formation of ice crystals inside of plant tissue. The location of ice crystals within tissues plays an important role in plant survival. Intracellular ice formation occurs when ice crystals form within the cytoplasm, which induces the malfunction of organelles and the cell membrane, thus resulting in cell death (Pearce and McDonald, 1977). Intracellular ice crystals generally form when the rate of temperature drop is greater than  $3^{\circ}\text{C}$  per hour (Palva, 1994). However, under natural conditions, the rate of freezing usually does not exceed  $2^{\circ}\text{C}$  per hour, which instead induces the formation of extracellular ice crystals mainly in apoplastic spaces (Levitt, 1980; Steffen et al.,



1989). The plasma membranes serves as a barrier between the ice crystals and the cytoplasm, which gives rise to a decreasing water potential gradient from the cytoplasm to extracellular spaces. In turn, prolonged presence of extracellular ice can result in dehydration and osmotic stress within the cytoplasm, which may potentially lead to the instability of cell membranes, denaturation of proteins, as well as the malfunction of cell organelles (Nilsen and Orcutt, 1996). However, unlike damage due to the presence of intracellular ice, extracellular ice-induced osmotic stress may be reversible once the cells are rehydrated.

In addition to development of osmotic stress, metabolic imbalances at freezing temperatures also result in the manifestation of oxidative stress. One of the major sources of oxidative stress at low temperature occurs in the chloroplasts of leaf tissues, specifically around the light reaction of photosynthesis. When light energy absorbed by chloroplasts exceeds the capacity for photosynthesis, oxygen molecules around the photosynthesis apparatus can be reduced to produce radical oxygen species (ROS) (Asada, 1996). In turn, ROS can readily diffuse through the cell and attack the cell membrane, proteins, and DNA, and other major cellular components, ultimately result in cell death.

### **Freezing Tolerance Mechanisms**

#### **Cold Acclimation**

Plants adapted to cold environments that routinely exposed to below-freezing temperatures have specialized mechanisms to survive extended period of extracellular ice formation in plant tissues, which is dependent on a period of cold acclimation during autumn months. Cold acclimation is induced by low but non-freezing and a decrease in photoperiod, light intensity and water availability, all of which typically

coincide with conditions of autumn and early winter (Mahfoozi et al., 2000; Fowler et al., 2001). In addition to exposure to non-freezing temperatures, enhanced freezing tolerance of certain temperate plants, including grasses, also require exposure to mild subfreezing temperature (Tumanov, 1940; Trunova, 1965; Herman et al., 2006), referred to as subzero acclimation or second phase cold hardening (Tumanov, 1940). Subzero acclimation will further improve freezing tolerance of plants, and this additional cold acclimation phase has been shown to be important for temperate grass species (Dionne et al., 2001; Espevig et al., 2011; Hoffman et al., 2010). Previous research has shown the rate of attaining maximal freezing tolerance during cold acclimation varies among different plant species (Chen et al., 1979; Fennell et al., 1985; Vega et al., 2000), ranging from a few weeks to months to achieve the maximum freezing tolerance. The capacity of plants to undergo cold acclimation and gain freezing tolerance depends on the recognition of environmental signals (including temperature and light), along with subsequent changes at the gene through whole plant levels to prepare cells to withstand prolonged freezing conditions.

Low temperature induces the expression of cold-regulated genes, generally referred to as *COR* genes (Weiser, 1970; Thomashow, 1990). Based on whole transcriptome studies in *Arabidopsis*, it has been demonstrated that approximately 1000 genes are either up- or down- regulated in response to low temperatures, which comprise approximately 4 to 20% of the *Arabidopsis* genome (Fowler and Thomashow, 2002; Lee et al., 2005; Hannah et al., 2005; Matsui et al., 2008; Zeller et al., 2009). The *COR* genes include members of the CBF (C-repeat binding factor)/DREB (dehydration responsive element binding protein) transcriptional factor family, which is currently the best characterized low temperature signaling pathway (Thomashow, 1999; Jaglo et al., 2001; Fowler and Thomashow, 2002; Yamaguchi-

Shinozaki and Shinozaki, 2006). Changes in CBF/DREB transcription factors have been shown to occur within minutes of low temperature exposure, leading to *COR* gene expression and increases in freezing tolerance (Yamaguchi-Shinozaki and Shinozaki, 1994; Gilmour et al., 1998; Jaglo-Ottoson et al., 1998; Fowler and Thomashow, 2002). In turfgrass species, CBF homologs have been identified and associated with freezing tolerance in perennial ryegrass (Xiong and Fei, 2006; Tamura and Yamada, 2007; Zhao and Bughara, 2008) and tall fescue (Tang et al., 2005). Although most research have focused on CBF/DREB pathways, these may not be the only transcription factors that control cold acclimation, and therefore additional research is needed to understand other pathways that may be involved in the expression of genes leading to improved freezing tolerance and overwintering capacity of plants (Chinnusamy et al., 2007).

Many of the *COR* gene products serve critical roles in protecting cells from freeze-induced dehydration, improving the stability of cell membranes at low temperature, preventing protein denaturation, and minimizing oxidative stress from ROS (Gilmour et al., 1992; Thomashow, 1999; Xin and Browse, 2000; Karpinski et al., 2002; Cook et al., 2004; Guy et al., 2008). Most studies examining physiological and biochemical changes of grass species during cold acclimation have reported, increased accumulation of non-structural carbohydrates (Patton et al., 2007a; Dionne et al., 2010; Espevig et al., 2011), induction of *COR* proteins such as late embryogenesis abundant (LEA) and antifreeze proteins (AFP) (Patton et al., 2007b; Zhang et al., 2009; Zhang et al., 2011), and modifications in lipid composition that enhance membrane fluidity and stability (Samala et al., 1998; Cyril et al., 2002; Munshaw, 2004; Hoffman et al., 2010). Differences in the capacity to modify these various physiological and biochemical traits during cold acclimation have been shown

to contribute to intra- and inter-specific differences in turfgrass freezing tolerance.

### **Cold Deacclimation Resistance**

In addition to traits associated with cold acclimation and the attainment of freezing tolerance, it is also critical to understand the underlying mechanisms required to plants to maintain freezing tolerance throughout winter months, particularly where fluctuations in temperatures or freeze-thaw cycles can increase the sensitivity of deacclimation of plant species. Cold deacclimation is herein defined as a loss of freezing tolerance that is generally triggered by increases in air and soil temperatures along with increases in photoperiod (Kalberer et al., 2006; Rapacz, 2002a,b). Deacclimation typically occurs in late winter through spring, where plants undergo metabolic and physiological changes for resumption of plant growth (Sasaki et al., 2001; Rapacz, 2002a; Arora et al., 2004). Although less research has been conducted on factors regulating deacclimation compared to cold acclimation, it has generally been observed that the metabolic changes occurring during deacclimation are more rapid compared to similar changes during cold acclimation (Gusta and Fowler, 1976; Rapacz, 2002b). For example, previous studies have reported that accumulated freezing tolerance attained during cold acclimation was lost within a few days to a week following deacclimation in some species (Levitt, 1980; Guy and Huskell, 1987). Therefore, if plants are exposed to elevated temperatures in mid-winter to early spring that trigger the deacclimation process, this may lead to untimely loss of freezing tolerance and low temperature kill when temperatures subsequently drop below freezing.

The potential for deacclimation and winter damage can be affected by the magnitude of temperature increase and the duration of exposure to elevated

temperatures (Gay and Eagles, 1991; Eagles and Williams, 1992; Svenning et al., 1997; Kalberer et al., 2006; Gu et al., 2008; Patgers and Arora, 2013; Hoffman et al., 2014a), as well as photoperiod (Rapacz, 2002b; Juntilla et al., 1997). Although temperature seems to be the primary factor triggering deacclimation (Patger and Arora, 2013), increases in photoperiod can exacerbate the deacclimation response (Eagles, 1994; Juntilla, 1997; Rapacz, 2002b). The extent of deacclimation and reacclimation capacity in response to mid-winter thawing events seem to be associated with whole-plant growth responses, such that deacclimation becomes irreversible under conditions where the plant initiates regrowth (Rapacz et al., 2001; Rapacz, 2002b).

Although relatively few studies have investigated genetics of the cold deacclimation responses, the available research suggests that genes up-regulated during cold acclimation tend to be down-regulated during deacclimation, and vice-versa (Cattivelli and Bartels, 1990; Nordin et al., 1991; Wolfrim et al., 1993; Oono et al., 2006; Welling and Palva, 2008). However, not all genes down-regulated during deacclimation were previously up-regulated during cold acclimation (Kalberer et al., 2006), such as the expression of the gene controlling RNA and protein metabolism in winter rye, which was further down-regulated from cold acclimation to deacclimation (In et al., 2005).

The altered gene expression at early stages of deacclimation promote changes at the cell to whole-plant levels, including changes in cellular water content, modifications in membrane lipid composition including increase in phospholipids saturation levels, and an increase in the metabolism of protective solutes (Tronsmo et al., 1993; Ögren, 1997; Arora et al., 2004; Iivonen et al., 2004; Patger and Arora, 2013). More specifically, deacclimation and loss of freezing tolerance has been associated with a decreased concentration of protective compounds including

carbohydrates, proteins, amino acids, and dehydrins (Svenning et al., 1997; Arora et al., 2004; Pagter et al., 2011).

Since climate warming results may result in a much milder winter in future years and mid-winter temperature fluctuations become more unpredictable (Arctic Climate Impact Assessment, 2005; Intergovernmental Panel on Climate Change, 2007), deacclimation of temperate plant species may become more of a problem in future years (Repo et al., 1996; Taulavuori et al., 2004; Pagter and Arora, 2013). Premature losses in freezing tolerance due to deacclimation has already been shown to contribute to significant losses in plant yield (Bokhorst et al., 2008; Ogren, 1996; Taulavuori et al., 1997; Zhu et al., 2000). Therefore, to better understand how to limit deacclimation, additional research is necessary to better understand the primary physiological factors responsible for differences in deacclimation resistance among plants.

### **Regulation of cold acclimation and deacclimation: Research gaps**

Carbon metabolism encompasses the processes associated with the assimilation and consumption of carbohydrates, including the light reactions and carbon reactions of photosynthesis, carbon allocation and partitioning, and respiration activities. A vast majority of studies evaluating carbon metabolism responses in relation to freezing tolerance have focused on the accumulation of total or individual carbohydrates in response to changes in temperatures. This may be attributed to the important role of carbohydrates in plant freezing tolerance, which include maintaining the integrity and fluidity of cell membranes, and protecting the organelles and cell function under freeze-induced dehydration (Santarius, 1982; Anchozdoguy et al., 1987; Alberdi and Corcuera, 1991; Steponkus, 1993). More recently, carbohydrates such as sucrose and fructans have been specifically reported to delay freezing by direct

inhibition in ice crystal growth in the apoplast (Livingston et al., 2009), and also play a role in regulation of the oxidative stress response at low temperature (Parvanova et al., 2004). In grasses, researchers have reported a relationship between freezing tolerance and either an increase in the total non-structural carbohydrate content during cold acclimation (Fry et al., 1993; Cai et al., 2004; Ball et al., 2002; Hoffman et al., 2010), or of individual carbohydrate fractions such as fructans (Dionne et al., 2010; Espevig et al., 2011) and (Fry et al., 1993; Ball et al., 2002; Zhang et al., 2006).

In contrast to the large accumulation of carbohydrates during cold acclimation, limited research suggests that deacclimation has been associated with a decrease in large pools of carbohydrates for some species (Morin et al., 2007; Pagter and Arora, 2013), which might account for the loss of freezing tolerance during deacclimation (Pagter et al., 2011). The mechanism under large changes in carbohydrate pools may be associated with temperature-induced changes in respiration, as it is well recognized that plant respiration rates are highly responsive to temperature fluctuations. In relationship to deacclimation, it has been shown that plant respiration rates were enhanced by mild winter temperatures, thus reducing carbohydrate pools and cold hardiness (Ögren, 1996; Ögren et al., 1997). The capacity to minimize winter respiration rates has been linked to improving winter survival of winter wheat (Sagisaka et al., 1991) and some cool-season forage grasses (Bertrand et al., 2003).

In addition to changes in respiration, photosynthesis capacity may also increase during deacclimation event (Öquist and Hunter, 2003; Bokhorst et al. 2010, Saarinen et al. 2011). The recovery rate of photosynthesis depends on the temperature and plant growth environment, with higher temperatures resulting in a much more rapid recovery of photosynthesis (Saarinen et al., 2011).

Although the significant emphasis related to carbon metabolism has been

placed on the accumulation and consumption of carbohydrates in relation to freezing tolerance, there is very little known on the actual regulation of carbon metabolism, particularly regarding energy utilization between the light reactions and the Calvin cycle, as well as respiratory acclimation at low temperatures for different turfgrass species. Moreover, we do not have a good understanding of how these carbon metabolism parameters may change in response to cold acclimation and deacclimation in grasses. For example, differences in capacity to minimize photoinhibition and recover photosynthesis during cold acclimation have been shown to contribute to intra- and interspecific differences in freezing tolerance for some species (Hurry et al., 1995; Pocock et al., 2001; Humphreys et al., 2007). Therefore, a better understanding of the photosynthetic and respiration characteristics among grasses with varying acclimation and deacclimation capacities may provide additional tools to be used to select better adapted grasses for northern environments.

Plant hormones are essential signaling molecules that regulate all aspects of plant growth and metabolism. There are five primary classes of plant hormones including abscisic acid (ABA), gibberellic acid (GA), cytokinins, ethylene, and auxin. In addition, salicylic acid (SA) and jasmonic acid (JA) have also been more recently categorized as plant hormones. Due to the widespread role of plant hormones, particularly in terms of abiotic and biotic stress resistances, it is not surprising that these signaling molecules have been reported to play a role in the development of plant freezing tolerance as described below.

Abscisic acid is involved in many plant metabolism activities including leaf senescence, bud dormancy, seed germination and maturation, protein synthesis, pathogen defense, osmotic adjustment and stomatal closure (Swamy and Smith, 1999; LaRosa, et al., 1987; Singh et al., 1989). Because of its role in water relations and



osmotic adjustment, ABA serves as a critical signal that mediates plant responses to different dehydration related stresses including drought, salt, and freezing stresses (Rikin et al. 1976; Chen et al., 1982; Gusta et al., 1982; Mohapatra et al., 1988; Davies and Zhang, 1991; Moons et al., 1995; Swamy and Smith, 1999; Alves and Setter, 2000; Schroeder et al., 2001). Increased concentration of ABA was observed during early stages of cold acclimation under both controlled environment and field conditions (Machakova et al., 1988; Taylor et al., 1990; Dörffling et al., 1990), suggesting that it could help to improve the freezing tolerance of plants (Rikin et al., 1979; Wrightman, 1979). In some cases, ABA can serve as a signal to trigger plant cold acclimation (Perras and Sarhan, 1989; Dörffling et al., 1990; Pearce, 1999). The maximum freezing tolerance of some plants was achieved when ABA concentration reached peak levels (Dörffling et al., 1990). In addition, exogenous application of ABA was also found to stimulate cold acclimation (Chen and Gusta, 1983) and increased freezing tolerance (Chen et al., 1979; Reaney and Gusta, 1987; Robertson et al., 1987; Guy and Huskell, 1988; Mohapatra et al., 1988; Dörffling and Askman, 1989; Luo et al., 1992; Lång et al., 1994). Some potential mechanisms for improvement of freezing tolerance may be related to the ABA-induced reduction in leaf elongation rates (Kende and Zeevaart, 1997; Rapacz et al., 2003), as well as enhanced protection of the photosynthesis machinery from oxidative stresses (Rapacz, 2002c). In addition, ABA has been found to participate in the regulation of cold-regulated (*COR*) gene expression of plants under stress conditions (Hajela et al., 1990; Lång and Palva, 1992; Mahajan and Tuteja, 2005). During deacclimation, both Rapacz et al. (2003) and Dörffling et al. (1990) reported increases in ABA concentration following a few weeks of deacclimation. However, during the early stages of deacclimation, declines in ABA content were reported in several species

such as bermudagrasses (Zhang et al. 2011), winter wheat (Taylor et al. 1990) and pea (Wealbaum et al., 1997).

Gibberellic acid is also an important hormone that is associated with stimulation of stem elongation and flowering, breaking seed dormancy and delaying senescence. In relation to freezing tolerance, increased concentration of GA resulted in greater damage to plants due to the increased susceptibility to photoinhibition (Rapacz, 2002c). Achard et al. (2008) reported that at low temperature, the up-regulation of C-repeat-binding transcription factors (CBFs) restrict plant growth rates by controlling the concentration of GAs. Rood et al. (1989) found a significant increase in GA concentration during the time when plants start to elongate stems and flower. Therefore, the reduction of freezing tolerance during deacclimation may be connected with GA due to its role in stimulating plant regrowth (Junttila, 1997). It has also been reported by Rapacz (2002c) that higher concentration of GA can result in much more rapid deacclimation in plants, and less cold acclimation and reacclimation capacity.

Cytokinins are well known as the antagonists of ABA based on their roles regulating plant growth and development activities (Blackman and Davies, 1984; Thomas, 1992). The major roles of cytokinins are related to cell division and expansion, stimulating primary root elongation, and delaying leaf senescence. The importance of cytokinins in relation to abiotic stress tolerance has been documented for heat stress (Veselov et al. 1995), drought stress (Lopez-Carbonell et al. 1996) and salinity stress (Kuiper et al., 1990). In respect to low temperature stress, Guo et al. (2010) reported that increased concentration of cytokinins, along with the expression of some genes leading to the development of freezing tolerance of plants. However, Taylor et al. (1990) compared the cytokinins levels of winter wheat during cold

acclimation and deacclimation and concluded that cytokinins did not respond to cold acclimation but played a more important role in deacclimation. In bermudagrass, increased concentrations of cytokinins was associated with higher photochemical efficiency and recovery (ie. green up) as temperature increased during deacclimation. (Zhang and Ervin, 2004; Zhang et al., 2011).

Auxin has major functions in plant development such as apical dominance, tropic responses, and root and shoot initiation (Davies, 1995). There are many interactions between auxin and other phytohormones, including antagonistic responses with cytokinins (Nordström et al., 2004) and ABA (De Smet et al.2003), and synergistic responses with ethylene (Morgan and Hall, 1962) and GA (Ross et al., 2000). Both auxin signaling and transport were shown to be impacted by low temperatures (Miura et al., 2011; Parry et al. 2006; Shibasaki et al., 2009), although a direct role of endogenous auxin for freezing tolerance has not been demonstrated. Applying exogenous auxin (TA-2 and TA-4) to rapeseed (*Brassica napus*) at low temperature stimulated the accumulation of proline and soluble sugars (Gavelienè et al., 2013), suggesting a potential role for improving freezing tolerance.

### **Freezing Tolerance of Cool-season Turfgrasses**

Cool-season turfgrasses have a C3 photosynthesis pathway, with optimal growing conditions at temperatures of 18 to 24 °C. Cool-season turfgrasses are well adapted in temperate regions with a cool humid and sub-humid climate (Hartley, 1950). There are more than 20 cool-season grasses that are widely used as turf (Beard, 1973), including *Agrostis spp.* (bentgrasses), *Poa spp.* (bluegrasses), *Lolium spp.* (ryegrasses), and *Festuca spp.* (fescues). Warm-season turfgrasses have a C4 photosynthesis pathway, and are adapted to tropical and subtropical regions with

temperatures of 27 to 35 °C. Common warm-season grasses used for turf are comprised of *Zoysia spp.* (zoysiagrasses), *Cynodon spp.* (bermudagrasses), *Eremochloa ophiuroides* (centipedegrass), and *Stenotaphrum secundatum spp.* (St. Augustinegrass).

Although cool-season turfgrasses are generally adapted to northern climates, the species do exhibit differences in freezing tolerance and overwintering capacities (Gusta et al., 1980). Among cool-season grasses used for turf, creeping bentgrass (*Agrostis stolonifera L.*) and Kentucky bluegrass (*Poa pratensis L.*) generally exhibit significantly higher freezing tolerance and thus lower susceptibility to winter injury, compared to species such as annual bluegrass (*Poa annua L.*) and perennial ryegrass (*Lolium perenne L.*) (Gusta et al. 1980, Dionne et al., 2001; Bertrand et al., 2013). Previous research also found that freezing tolerance can significantly vary among different cultivars or genotypes within the same species (Gusta et al., 1980; Ebdon et al., 2002; Hulke et al., 2007; Hoffman et al., 2010). It is widely accepted that freezing tolerance is one of the most important parameters determining winter hardiness of plants (Humphreys and Eagles, 1988; Hulke et al. 2008). Because of the poor winterhardiness of some grass species, winterkill occurs on golf courses and home lawns as a major problem during winter months, resulting in compromised turf quality, function and economic loss. Therefore, in order to improve turfgrass freezing tolerance and overwintering capacity, a better understanding of the genetic and physiological mechanisms associated with cold acclimation and deacclimation is essential.

## **Biology of Cool-season Turfgrasses Differing in Freezing Tolerance**

### **Annual Bluegrass**

Annual bluegrass is a cool-season grass species that is widely prevalent in temperate regions (Warwick, 1979; Huff, 1996). In northern climates, annual bluegrass is usually recognized as an undesirable turf species due to its sensitivity to a number of biotic and abiotic stresses, however, due to its aggressiveness in turf stands and depending on cultural practices, this species can comprise large areas of golf course turf particularly on greens, tees, and fairways. In some instances, annual bluegrass has the capacity to exhibit very fine leaf texture, high density and tolerance to low heights of mowing, thus turfgrass managers often debate on whether to maintain this species as a desired turfgrass or eradicate it as a weed (Johnson et al. 1993).

Annual bluegrass are categorized according to biotypes or ecotypes, and generally consist of annual and perennial types (Johnson et al., 1993). Different biotypes have different biological traits. The annual type of annual bluegrass (*Poa annua* f. *annua* L.) is considered to exist as winter annual and its habitat is primarily in warm climate zones such as the southern part of the United States (Huff, 2003). It is a bunch type grass that germinates in spring or fall, but dies at supra-optimal temperatures experience in summer months. The perennial biotype of annual bluegrass (*Poa annua* f. *reptans* L.) is a perennial grass species and widely spread in cool climates. It has a creeping, prostrate growing habit that produces large amounts of tillers which increase the density of the turf (Hovin, 1957). The perennial biotype of annual bluegrass is commonly found on highly maintained turf such as golf course putting greens (Huff, 2003).

Annual bluegrass is very susceptible to environmental stresses especially

extremes in temperature (Beard et al., 1978), and different annual bluegrass biotypes exhibit different stress tolerances (Tompkins et al., 2000; Dionne et al., 2001; Tompkins et al., 2004; Hoffman et al., 2014a). Annual bluegrass also exhibits high susceptibility to important diseases such as dollar spot (*Sclerotinia homoeocarpa*), brown patch (*Rhizoctonia solani*), anthracnose (*Colletotrichum cereal*), summer patch (*Magnaporthe poae*) and red thread (*Laetisaria fuciformis*) (Vargas, 1994).

### **Creeping Bentgrass**

Creeping bentgrass (*Agrostis stolonifera*, L.) is a perennial cool-season grass species with stoloniferous growing habit. It is tolerant to close mowing and high maintenance thus is widely selected on golf courses, home lawns, and sports fields (Warnke, 2003). Creeping bentgrass is mainly adapted to cool climates and widely utilized in temperate regions, creeping bentgrass is also a popular golf course putting green grass species due to its high turf quality characteristics (Warnke, 2003).

Creeping bentgrass is known to have superior freezing tolerance than most of other turf species (Beard, 1973). This species also exhibits relatively good tolerance to other abiotic stresses such as flooding and salinity stresses (Warnke, 2003). However, creeping bentgrass is susceptible to multiple turfgrass diseases including dollar spot (*Sclerotinia homoeocarpa*), brown patch (*Rhizoctonia solani*), and pink snow mold (*Microdochium nivale*) (Warnke, 2003). Different creeping bentgrass cultivars may exhibit different stress tolerance and disease resistance (Marcum, 2001; Warnke, 2003; Jiang and Wang, 2006; Bonos et al., 2006).

### **Perennial ryegrass**

Perennial ryegrass (*Lolium perenne*) is a grass species that highly cultivated

for forage, turf and bioenergy uses (Thorogood, 2003). It is widely found in Europe, Asia, North America, and Oceania. Perennial ryegrass exhibits a bunch type growth habit with good turf quality, high canopy density, high wear tolerance and quick establishment traits. It is commonly used on landscape areas, home lawns, sports fields, as well as golf courses.

Perennial ryegrass has origins in Europe, therefore it is well adapted to cool, humid regions (Beard, 1973; Thorogood, 2003). However, perennial ryegrass exhibits sensitivity to temperature extremes and water stress, such as heat, freezing, and drought stresses (Van Dersal, 1936; Gusta et al., 1980; Thorogood, 2003; Turgeon, 2005). In addition, perennial ryegrass is susceptible to brown patch (*Rhizoctonia solani*), red thread (*Laetisaria fuciformis*), dollar spot (*Sclerotinia homoeocarpa*), *Pythium* blight (*Pythium aphanidermatum*), grey leaf spot (*Pyricularia grisea*), and crown rust (*Puccinia coronata*) (Thorogood, 2003). Because of its good performance for turf, forage and bioenergy uses, breeders have placed significant efforts in the development of new cultivars with good abiotic and biotic stress characteristics (Humphreys and Eagles, 1988; Hulke et al., 2007; Bonos et al., 2009; Morris, 2009). To date, there are more than perennial ryegrass cultivars and 200 of them are commonly seen on market (Bonos, 2007).

### **Research Justification**

Freezing tolerance is a major component of winter hardiness of plants (Humphreys and Eagles, 1988; Xiong and Fei, 2006), and can be enhanced through the process of cold acclimation (Levitt, 1980). However, even if plants have undergone optimal cold acclimation going into winter months, damage may still occur due to the process of deacclimation in response to fluctuating temperatures. In response to

deacclimation, plants may start to take up water, rehydrate the cells, and up-regulate plant metabolism in order to return to normal growth. However, if plants are once again exposed to freezing temperatures, the cells may no longer have the same capacity to resist extracellular or intracellular ice formation. Therefore, in order to reduce the damages brought by winter injury, a better understanding of the underlying factors that are responsible for the loss of freezing tolerance during deacclimation is necessary. In addition, it would be useful to better understand the relationship between cold acclimation capacity and cold deacclimation resistance. For example, it has been demonstrated that plants that exhibit high cold acclimation capacity do not necessarily exhibit high deacclimation resistance (Hummer et al., 1986; Rowland et al., 2005; Arora and Rowland, 2011; Pagter et al., 2011) To date, this relationship in turfgrass has not been extensively evaluated.

For our studies, we will evaluate freezing tolerance mechanisms of cool-season grasses with contrasting winter injury potential, including freezing tolerant creeping bentgrass, and freezing sensitive annual bluegrass and perennial ryegrass. Annual bluegrass is widely found on golf course greens, tees, and fairways in the northern U.S. and Canada. Previously published research using both field and controlled environmental conditions have reported that creeping bentgrass exhibits a greater freezing tolerance capacity than annual bluegrass following cold acclimation and deacclimation (Beard, 1966; Tompkins et al., 2000; Tompkins et al., 2004; Hoffman et al., 2014a,b). However, the underlying factors associated with the differences in freezing tolerance remains unknown. Perennial ryegrass is an important temperate grass species that widely selected for turf, forage and even bioenergy uses (Jung et al., 1996). However, this species can experience significant winterkill (Gusta et al., 1980; Bertrand et al., 2013). Due to its important roles in turf, forage, and bioenergy uses, breeders are



dedicated to cultivate genotypes that perform better freezing tolerance. The genotypes and cultivars of perennial ryegrass vary greatly in freezing tolerance (Ebdon et al., 2002; Hulke et al., 2007). For a better understanding of the factors responsible for differences in freezing tolerance of perennial ryegrasses and provide more information for breeders in the future, a close look into the physiological changes during cold acclimation and deacclimation is necessary.

### **Objectives and Hypotheses**

The primary objectives of this research are to examine mechanisms responsible for freezing tolerance differences among cool-season turfgrasses, with a focus on cold acclimation and deacclimation. To address this goal, we proposed to conduct four controlled-environment studies to better understand the physiological mechanisms of freezing tolerance, as follows:

- I. Evaluate the carbon metabolism factors attributing to different freezing tolerance capacity associated with cold acclimation and deacclimation of annual bluegrass and creeping bentgrass;
- II. Investigate the differences in hormone regulation of annual bluegrass and creeping bentgrass during cold acclimation and deacclimation;
- III. Compare the freezing tolerance of eight different genotypes perennial ryegrass;
- IV. Identifying the physiological changes in response to cold acclimation and deacclimation among perennial ryegrass genotypes contrasting in freezing tolerance

Based on previous research, we hypothesized that different grass species may exhibit different cold acclimation and deacclimation capacities. For the grasses with lower

deacclimation resistance, metabolic activities will be up-regulated faster in response to warm temperature than the species with higher deacclimation resistance.

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## CHAPTER 2

### CARBON METABOLISM RESPONSES ASSOCIATED WITH COLD ACCLIMATION AND DEACCLIMATION OF CREEPING BENTGRASS AND ANNUAL BLUEGRASS

#### Abstract

Winter injury on turfgrasses induced by mid-winter deacclimation events is a significant concern in northern climatic regions. To reduce turf stand losses and associated recovery costs, a better understanding of the underlying factors associated with the loss of freezing tolerance in response to premature deacclimation is necessary. Therefore, the objectives of this study were to: (i) compare the freezing tolerance of annual bluegrass (*Poa annua* L.) (AB) and creeping bentgrass (*Agrostis stolonifera* L.) (CB) during acclimation and deacclimation; and (ii) examine carbon metabolism changes for these two species as related to cold acclimation and deacclimation. Plants were exposed to five temperature treatments in a controlled environment growth chamber as follows: (1) non-acclimated control at 20 °C for 2 weeks; (2) cold acclimated at 2 °C for 2 weeks; (3) cold acclimated at -2 °C for 2 weeks; (4) deacclimated at 8 °C for 1 d; and (5) deacclimated at 8 °C for 5 d. For each treatment, changes in freezing tolerance were evaluated based on lethal temperature resulting in 50% kill (LT<sub>50</sub>). In addition, carbon metabolic activities including photosynthesis, respiration, and chlorophyll fluorescence parameters, including photochemical efficiency (F<sub>v</sub>/F<sub>m</sub>) and photochemical yield (Yield), electron transport (ETR) and non-photochemical quenching (NPQ) were measured following each treatment. Overall, CB exhibited higher freezing tolerance (lower LT<sub>50</sub>) following cold acclimation, and maintained higher freezing tolerance under deacclimation conditions compared to AB, as exhibited by a lower LT<sub>50</sub> at 8 °C for 1 and 5 d. Photosynthesis,

respiration,  $F_v/F_m$  and Yield of AB increased more rapidly during deacclimation, suggesting that the metabolic and physiological activities of AB are activated earlier in response to temperature increases, which may help to explain the potential differences in winter injury susceptibility between AB and CB.

## **Introduction**

In temperate regions, winter injury has been cited as a significant environmental stress limiting the quality and productivity of many plant species, including grasses used for turf and forage (Fry, 1990; Anderson et al., 1997; Dionne et al., 1999; Bélanger et al., 2002). Although most research addressing issues with predicted increases in future global temperatures have focused on high temperature effect on crop plants in summer months, warming events that occur in the fall, winter, and/or spring may have significant impact on plant sensitivity to freezing damage (Cannell and Smith, 1986). Elevated temperatures may negatively impact survival by prolonging the fall growing season and prevent maximal cold hardening prior to freezing temperatures, while more frequent temperature fluctuations throughout winter months (freeze-thaw events) may result in premature losses in freezing tolerance (Bélanger et al., 2002; Thorsen and Höglind, 2010). Consequently, it is important to understand plant and environmental factors affecting the capacity of plants to attain and maintain freezing tolerance throughout winter months.

Plants adapted to cold environments that are regularly exposed to below-freezing temperatures have specialized mechanisms to survive extended periods of extracellular ice formation in plant tissues, which is dependent on a period of cold acclimation during autumn months. Cold acclimation is induced by low, non-freezing temperatures and a decrease in photoperiod, light intensity and water availability (Mahfoozi et al., 2000; Fowler et al., 2001). Additional exposure to mild subfreezing temperatures, referred to as subzero acclimation, can further improve freezing tolerance in temperate grasses (Dionne et al., 2001; Espevig et al., 2011; Hoffman et al., 2010). Cold acclimation occurs as the result of the expression of cold-regulated genes, which increase the presence of metabolites that serve critical roles in protecting

cells from freeze-induced dehydration, improving the stability of cell membranes at low temperature, and minimizing oxidative stress from reactive oxygen species (ROS) (Gilmour et al., 1992; Thomashow, 1999; Xin and Browse, 2000; Karpinski et al., 2002; Cook et al., 2004; Guy et al., 2008). The rates of cold acclimation vary among different plant species, ranging from a few weeks to months to achieve maximal freezing tolerance, which can be significantly impacted by environmental conditions and agronomic practices (Chen et al., 1979; Fennell et al., 1985; Vega et al., 2000).

In contrast, cold deacclimation is associated with increases in air and soil temperatures along with increases in photoperiod that lead to progressive or rapid losses in freezing tolerance (Levitt, 1980; Kalberer et al., 2006; Rapacz, 2002a,b). Deacclimation typically occurs in late winter through spring, where plants undergo metabolic and physiological changes to favor resumption of plant growth (Sasaki et al., 2001; Rapacz, 2002a; Arora et al., 2004). Although less research has been conducted on factors regulating deacclimation compared to cold acclimation, deacclimation is reported to occur more rapidly compared to cold acclimation (Gusta and Fowler, 1976; Chen and Li, 1980; Gay and Eagles, 1991). For example, previous studies have reported that accumulated freezing tolerance attained during cold acclimation was lost within a few days to a week following deacclimation in some species (Levitt, 1980; Guy and Huskell, 1987; Hoffman et al., 2014a). Therefore, plants exposed to elevated temperatures in mid-winter to early spring may trigger the deacclimation process and lead to premature losses of freezing tolerance and low temperature kill when temperatures subsequently drop below freezing.

Most studies examining physiological and biochemical changes of grass species during cold acclimation have reported changes in the accumulation of total or individual carbohydrates in relation to freezing tolerance (Fry et al., 1993; Ball et al.,

2002; Cai et al., 2004; Zhang et al., 2006; Patton et al., 2007; Dionne et al., 2010; Hoffman et al., 2010; Espevig et al., 2011). This is due to the important role of carbohydrates in plant freezing tolerance, which include maintaining the integrity and fluidity of cell membranes, and protecting the organelles and cell function under freeze-induced dehydration (Santarius, 1982; Anchordoguy et al., 1987; Alberdi and Corcuera, 1991; Steponkus et al., 1993). However, very few studies have investigated the regulation in overall carbon metabolism pathways that ultimately influence the accumulation and metabolism of carbohydrates at low temperatures. Carbon metabolism encompasses the processes associated with the assimilation and consumption of carbohydrates, including the light reactions and Calvin cycle of photosynthesis, carbon allocation and partitioning, as well as respiration rates. There is not a clear understanding of how these carbon metabolism parameters may change in response to low temperatures and freeze-thaw cycles, and whether variations in these carbon metabolism parameters may lead to differences in cold acclimation capacity and deacclimation sensitivity in turfgrasses. For example, differences in the capacity to minimize photoinhibition and recover photosynthesis during cold acclimation have been shown to contribute to intra- and interspecific differences in freezing tolerance for some plant species (Hurry et al., 1995; Pocock et al., 2001; Humphreys et al., 2007). In addition, the ability to regulate carbon metabolism, such as respiration rates, has also been demonstrated to influence winter survival capacity (Sagisaka et al., 1991; Bertrand et al., 2003). Therefore, a better understanding of the photosynthetic and respiration characteristics among grasses with varying acclimation and deacclimation capacities may provide additional tools to be used to select better adapted grasses for northern environments.

In the northern United States and Canada, low temperature injury and

winterkill are considered major limitations in the management of particular cool-season turfgrasses used as golf turf, including AB. In contrast to golf greens, tees, and fairways composed primarily of CB, a species that generally demonstrates very good freezing tolerance, turf stands comprised of considerable populations of AB may experience as much as 70-90% turf loss from winterkill depending on environmental and cultural conditions (Skorulski, 2002). Investigations conducted under both field and controlled environmental conditions have shown that CB exhibits a greater freezing tolerance capacity compared to AB following a period of cold acclimation (Tompkins et al., 2000; Tompkins et al., 2004; Hoffman et al., 2014b). Moreover, research by Hoffman et al. (2014a,b) also demonstrated a greater potential for more rapid deacclimation of AB in response to elevated temperatures compared to CB that was associated with changes in carbohydrate and nitrogen metabolites. The goal of the current study was to expand on our previous research and gain a more thorough understanding of carbon metabolism pathways that may regulate differences in cold acclimation and deacclimation sensitivity among AB and CB. Therefore, the specific objective was to examine changes in plant photosynthesis, respiration, and chlorophyll fluorescence traits in relationship to freezing tolerance during cold acclimation and deacclimation.

## **Materials and Methods**

### **Plant Materials and Growing Conditions**

Plugs of one creeping bentgrass cultivar (Penncross) (Joseph Troll Turf Research Center, South Deerfield, MA) and one annual bluegrass ecotype (Silver Spring Country Club, Ridgefield, CT) were removed from the field and transplanted into containers (5 cm diameter, 25 cm depth) filled with sand. Plants were grown in a

greenhouse at 23 °C/18 °C (day/night) temperatures, irrigated three times per week, trimmed to 1 cm height of cut and fertilized with full strength Hoagland solution (Hoagland and Arnon, 1950) on a weekly basis. After establishing for 3 months in the greenhouse, plants were moved into a controlled environment growth chamber (Conviron, Winnipeg, CA) and maintained at 20 °C under a 10-hour photoperiod with a photosynthesis photon flux density (PPFD) of 300  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and a relative humidity of 50%.

### **Treatments**

Plants were exposed to cold acclimation and deacclimation treatments in the growth chamber that consisted of a total of five temperature treatments as follows: (i) non-cold acclimated control maintained at 20 °C for 2 weeks; (ii) cold acclimation at 2 °C for two weeks; (iii) sub-zero cold acclimation at -2 °C for two weeks; (iv) cold deacclimation at 8 °C for 1 day, and (v) cold deacclimation at 8 °C for 5 days. The deacclimation regime of 8 °C was selected based on the research conducted by Hoffman et al. (2014a), which demonstrated this temperature to induce deacclimation for both AB and CB. The light level of the growth chamber was maintained at 300  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD for cold acclimation treatments and at 150  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD for deacclimation treatments. Plants were maintained in the dark during the sub-zero acclimation period at -2 °C to simulate natural conditions under snow. At the end of each treatment period, plants were harvested for freezing tolerance assessment and carbohydrate analysis as described below.

### **Freezing Tolerance Assessment ( $LT_{50}$ )**

Freezing tests were conducted immediately at the end of each temperature



treatment to assess changes in freezing tolerance during cold acclimation and deacclimation. Ten individual plants (leaf, crown and 1 cm of roots) per replicate were wrapped in moist paper towels and placed into plastic bags for each temperature treatment according to the methods described by Ebdon et al. (2002). All bags containing plant materials were held at 2 °C for the duration of the harvest. Plants were exposed to six freezing temperatures in a programmable freezing chamber (Tenney TC Series Cycling Test Chamber, SPX Thermal Product Solutions, White Deer, Pennsylvania) as follows: -6, -9, -12, -15, -18, and -21 °C. The freezer was programmed to decrease at a rate of 2 °C per hour, along with 1 hour plateau at each freezing test temperature. After the freezing test, plants were transplanted into the cell trays containing commercial potting media (Pro-mix; Griffin Greenhouse and Nursery Supplies, Tewksbury, MA) and maintained in the greenhouse at 23/18 °C (day/night) for recovery. Following three weeks, the survival percentage at each freezing temperature was evaluated by counting the number of living plants out of the total number of plants. The LT<sub>50</sub> of each genotype was calculated by fitting percent survival to temperature using the PROC LOGISTIC procedure.

### **Measurements**

In order to evaluate the carbon metabolism changes during cold acclimation and deacclimation periods, chlorophyll fluorescence parameters, canopy photosynthesis, and respiration were measured following two weeks at each treatment temperature at 20, 2, and -2 °C, or at 1 and 5 d at 8 °C.

For chlorophyll fluorescence, the quantum yield (Y), photochemical efficiency (Fv/Fm), electron transport rate (ETR), and non-photochemical quenching (NPQ) were measured with a portable chlorophyll fluorometer (Opti-Science Inc., Hudson,

NH). The fluorescence chamber was placed on the canopy of the plants at two locations per pot, and then averaged. Specifically,  $F_v/F_m$  was measured following a dark adaptation period of 30 min and calculated as  $(F_m - F_0)/F_m$ , which provided the ratio of variable fluorescence to maximum fluorescence. The  $F_0$  refers to the minimal fluorescence level measured by applying the modulated weak beam on the canopy while  $F_m$  refers to the maximum fluorescence level under saturated beam. The other two fluorescence measurements ( $Y$  and NPQ) were conducted under the ambient light levels, and NPQ was calculated based on the following formulas:  $NPQ = (F_m - F_{ms})/F_{ms}$ , where  $F_{ms}$  refers to the maximum fluorescence in a steady fluorescence condition.

Canopy photosynthesis and respiration rates were measured using a portable gas exchange system (CIRAS-2, PP Systems International, Inc., Amesbury, MA). The measurement was conducted by placing plexi-glass chamber to enclose the plant canopy, with the canopy chamber set to provide a constant  $CO_2$  concentration of  $400 \mu\text{mol mol}^{-1}$ . The light intensity at the turf canopy was  $300 \mu\text{mol mol}^{-1} \text{s}^{-1}$  that was provided by lights inside of the growth chamber. Respiration rate was measured in the same manner as described for photosynthesis, but measurements were conducted once plants were maintained under dark conditions for a minimum of one hour.

### **Carbohydrate Analysis**

Plants were harvested at the end of each temperature treatment to extract and quantify the carbohydrate content of crowns according to the methods of Ting et al. (1959) with modifications. For preparation, crowns were collected and dried at  $70^\circ\text{C}$  for a minimum of 72 h prior to being ground using a mortar and pestle. Approximately 50 mg of ground tissues were weighed into glass test tubes and mixed

with 2.5 ml amylase solution. The tissues were incubated at 37°C for 24 h. Next, 0.5ml 0.6N HCL (1:1 v/v) was added to the solution, mixed thoroughly, and incubated for an additional 18 h at room temperature. Lastly, 0.31 ml of 10N NaOH was added to each sample, and volumes of each tube were brought up to 10 ml with distilled water.

For total soluble carbohydrate analysis, 1 ml of extraction solution was mixed with 1.5 ml alkaline ferricyanide reagent and vortexed thoroughly. The mixture was boiled in a water bath for 10 min and then rapidly cooled to room temperature prior to adding 3 ml of 2N H<sub>2</sub>SO<sub>4</sub>. The solution was vigorously shaken to release all gas. Next, 1.2 ml arsenomolybdate solution was added and the solution was diluted to 10 ml with distilled water. The absorbance was measured at 515 nm with a spectrophotometer (Genesys 2, Thermo Electron LLC.; Madison, WI), and the total soluble carbohydrate content was calculated based on comparison to a glucose standard curve.

### **Experimental Design and Statistical Analyses**

Pots were completely randomized in the growth chamber, with a total of five temperature treatments and 4 replicates for each species-treatment combination. The data were analyzed using analysis of variance (ANOVA) according to the linear model procedure for the Statistical Analysis System v. 9.2 (SAS Institute, Inc. Cary, NC) and the means were separated with Fisher's protected least significant difference (LSD) test at the 0.05 probability level. The main effects of two species, five temperature regimes, and their interaction were analyzed by partitioning the total treatment sums of squares (SS) into single degree of freedom (df) orthogonal contrasts. Contrasts for species included the main effect of AB versus CB, while temperature regime included four orthogonal linear contrasts for comparing (i) mean

of non-acclimated versus the combined mean for all acclimation and deacclimation treatments; (ii) mean of acclimation at 2 and -2 °C versus the combined mean for deacclimation at 8 °C for 1 and 5 d; (iii) mean of acclimation at 2 versus -2 °C; and (iv) mean for deacclimation at 8 °C for 1 versus 5 d. Species and temperature regime main effects and associated contrasts were crossed to partition species × temperature regime interaction SS to test for single df interaction components.

## **Results and Discussion**

The results from the ANOVA for main effects and their interactions are presented in Table 2.1. The main effect of species was a significant factor influencing freezing tolerance ( $LT_{50}$ ), photosynthesis rate, and the chlorophyll fluorescence parameters of photochemical efficiency ( $F_v/F_m$ ), photochemical yield ( $Y$ ), and electron transport rate (ETR). In contrast, the main effect of temperature regime was highly significant for all measured parameters, and contributed to the greatest treatment variation among the data. There were also significant species × temperature interactions observed for all parameters except for total soluble sugar content. Additional discussion on the main effects, interactions, and orthogonal contrasts are detailed in subsequent sections.

Our results agree with previous reports on the higher cold acclimation capacity and freezing tolerance for CB compared to AB (Tompkins et al., 2000; Tompkins et al., 2004; Espevig et al., 2014; Hoffman et al., 2014b). Although the freezing tolerance of AB was found to be higher (lower  $LT_{50}$ ) than CB under non-cold acclimating conditions, similar to the results of Hoffman et al. (2014a) and Tompkins et al. (2000), successive cold acclimation at 2°C and -2°C resulted in higher freezing tolerance for CB ( $LT_{50}$  of -16.3°C) compared to AB ( $LT_{50}$  of -13.5°C) (Fig. 2.1).

When plants were exposed to deacclimation at 8°C, the freezing tolerance of both species significantly decreased compared to the baseline freezing tolerance at -2°C, even as early as 8°C for 1 d. For example, the freezing tolerance of AB and CB declined from -13.5 to -11.0°C and from -16.3 to -12.5°C, respectively, at 1 d of deacclimation. Additional freezing tolerance was lost by 5 d of deacclimation for both species, although CB maintained a higher freezing tolerance at both 1 and 5 d of deacclimation compared to AB. Overall the data suggest a higher rate of deacclimation for CB when exposed to temperatures of approximately 8°C, which is in agreement with published field and controlled environment experiments (Tompkins et al., 2000; Espevig et al., 2014; Hoffman et al., 2014a). Our lab previously reported that deacclimation rates for AB and CB varied depending on the deacclimation temperature (4, 8, and 12 °C) and duration (1 or 5 d) (Hoffman et al., 2014a). Acclimated AB exhibited a 2.5-fold greater loss in freezing tolerance compared to CB at 4 °C, whereas CB exhibited a 3-fold greater sensitivity and loss in freezing tolerance in response to longer exposures at higher temperatures. Similarly, Espevig et al. (2014) compared the deacclimation rates of several cool-season grasses and found that CB and AB deacclimated to a greater extent than other species when exposed to 10 °C, with CB exhibiting a more rapid loss of freezing tolerance compared to AB upon exposure to 10 °C for 6 d.

The development of freezing tolerance during cold acclimation requires a significant investment of energy to support the many metabolic changes required to prepare cells to survive prolonged freezing temperatures. As such, maximal cold acclimation and freezing tolerance has been positively associated with photosynthetic capacity and resistance to photoinhibition (Hurry and Hunter, 1991; Öquist et al., 1993; Krivosheeva, et al., 1996). Low temperatures can inhibit photosynthesis and

carbon assimilation by affecting components such as the structural integrity of membranes and photosystems, interfering with electron transport, and reducing the energy utilization through the carbon reduction reactions. Differences in the capacity to minimize photoinhibition and recover photosynthesis during cold acclimation have been shown to contribute to intra- and interspecific differences in freezing tolerance. (Hurry et al., 1995b; Pocock et al., 2001; Rapacz et al., 2004; Humphreys et al., 2007; Rapacz et al., 2007).

Evaluation of overall carbon metabolism in this study was conducted through measurements of chlorophyll fluorescence, gas exchange-based photosynthesis and respiration rates, as well as carbohydrate content of crowns. Chlorophyll fluorescence has been used as a rapid, non-invasive tool to indirectly monitor changes in photosynthesis capacity in response to abiotic stresses, including low temperature (Lichtenthaler and Rinderle, 1988; Schreiber and Bilger, 1993; Clement and van Hasselt, 1996; Rizza et al., 2001; Ehlert and Hinch, 2008; Mishra et al., 2011). Photochemical efficiency ( $F_v/F_m$ ) is a chlorophyll fluorescence parameter that provides information on the maximum potential quantum yield of Photosystem II (PSII) within the light reactions of photosynthesis, and is a good indicator how well absorbed light energy can be used for photosynthesis. In contrast to  $F_v/F_m$  that is measured under dark-adapted conditions, the effective quantum yield ( $Y$ ) is measured under steady-state light conditions and serves as an indicator of how well energy can move beyond PSII. Lastly, NPQ is generally comprised of a protective pathway to dissipate excessive excitation energy absorbed in the light harvesting system to prevent photooxidative damage, in addition to component of NPQ that may also be indicative of photoinhibitory damage to PSII (Müller et al., 2001).

We found that both  $F_v/F_m$  and  $Y$  significantly decreased for both species

during cold acclimation (Fig. 2.2 and 2.3). These results are in agreement with previously published research showing a similar decline in Fv/Fm during cold acclimation for creeping bentgrass, Kentucky bluegrass, and perennial ryegrass (Sarkar et al., 2009; Hoffman et al., 2010). When comparing the two species, CB exhibited higher Fv/Fm (0.64) compared to AB (0.58) following 2°C acclimation, whereas no species differences were observed at -2°C (Fig. 2.2). Similar responses were observed for Y (Fig. 2.3). This suggested that CB maintained higher capacity for absorbing light and moving energy through the photochemistry pathway; however, there were no differences detected in actual CO<sub>2</sub> assimilation rates as described in further detail below.

When plants were moved from -2°C (dark conditions) to 8°C for 1 d (light conditions), CB exhibited a significant decline in Fv/Fm, whereas that of AB remained at similar levels to what was observed at -2°C (Figure 2.2). While the Fv/Fm of AB significantly increased by 3 d of deacclimation, Fv/Fm activity was not restored in CB until plants were maintained at 8°C for 5 d. Moreover, Y was also significantly reduced for CB during the deacclimation period to a greater extent than that of AB (Fig. 2.3). Altogether this suggested that the photosynthetic apparatus of CB was less functional in response to the temperature shift from -2°C to 8°C, particularly at the shorter duration of 1 to 3 d. Moreover, Fv/Fm recovered more rapidly during deacclimation compared to Y regardless of species.

The decreased Fv/Fm and Y of CB during deacclimation was also associated with higher NPQ (Fig. 2.4). For example, although an increase in NPQ was observed for both species, CB exhibited a significantly higher NPQ (2.02) compared to AB (1.66) within 1 d of deacclimation. Together, higher NPQ along with lower Fv/Fm and Y at early stages of deacclimation indicate that the light harvesting systems or

protein-membrane complexes in CB may be down-regulated or damaged, which can inhibit the recovery of photosynthetic activity during short-term warming events. Under more prolonged deacclimation (8°C at 3 and 5 d), AB exhibited a higher NPQ compared to CB, although the NPQ at 8°C at 3 d dropped compared to what was observed at 1 d. Similar results were seen for winter barley (*Hordeum vulgare* L.), where NPQ significantly increased 18 h following freezing at -8°C but then decreased by 3 days of recovery (Dai et al., 2007). The reason behind these variable changes in NPQ remains unclear. Further detailed investigation regarding the NPQ pathways (photoprotection vs damage) may shed additional light on the role of this mechanism in cold acclimation, deacclimation, and the relationship with freezing tolerance.

Although a decline in Fv/Fm and Y were observed in response to acclimation at 2°C, photosynthesis rates were similar at 20 and 2°C, with rates at approximately 0  $\mu\text{mol m}^{-2} \text{s}^{-1}$  while plants were maintained in the dark at -2 °C (Fig. 2.5). Previous studies on cool-season forage grasses have shown that minimum temperatures for net photosynthesis can occur down to -4 °C, depending on light levels and exposure to frost conditions (Skinner et al., 2007; Höglind et al., 2011). No significant differences in photosynthesis rates were detected between AB and CB at 20, 2, and -2°C (Fig.2.5). In contrast, when plants were exposed to 8°C for 1 d, the photosynthesis rate of AB was found to be twice as high compared to CB, which was statistically equal to 0  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . As deacclimation progressed, the photosynthesis rate of both species increased, with AB maintaining significantly higher CO<sub>2</sub> assimilation compared to CB. In woody species, early activation of photosynthesis machinery during deacclimation was associated with high demands of photosynthates for bud burst and stem elongation (Repo et al. 2006, Hansen and Beck, 1994). Espevig et al. (2011) recently reported that AB exhibited significantly higher leaf growth rates compared to CB



following exposure at 10°C for 12 d. Although we did not measure leaf elongation in our current study, it is plausible that greater elongation rates of AB in response to warming events would require functional photosynthetic machinery to support continued growth, in addition to the use of reserve carbohydrates.

Plants consume photosynthates through respiration to provide energy and carbon structures for growth and maintenance processes. In response to cold acclimation, the respiration rate declined similarly for both species (Fig. 2.6). This is contrary to previous reports that respiration rate increased following a prolonged period cold acclimation (Klikoff, 1968; Atkin et al. 2000; Talts et al., 2004). When plants were maintained at -2°C, the respiration rate of both species was approximately  $0 \mu\text{mol} \cdot \text{m}^{-2} \text{s}^{-1}$ . During deacclimation, a significant increase in respiration rate for both species was observed, with the respiration rate of AB ( $1.14 \mu\text{mol} \cdot \text{m}^{-2} \text{s}^{-1}$ ) significantly higher than CB ( $0.86 \mu\text{mol} \cdot \text{m}^{-2} \text{s}^{-1}$ ) at 8°C 1 d. By 5 d, however, there was no difference in respiration rate between the two species.

Cold acclimation is generally associated with a decline in plant growth rate and phloem transportation activities, and increases the soluble sugar concentration in the source leaves (Guy et al., 1992; Strand et al., 1999). Many studies have reported a close association between turfgrass freezing tolerance and higher accumulation of total sugars following cold acclimation (Fry et al., 1993; Ball et al., 2002; Cai et al., 2004; Hoffman et al., 2010), or of individual carbohydrate fractions such as fructans, sucrose, and starch (Fry et al., 1993; Ball et al., 2002; Zhang et al., 2006; Dionne et al., 2010; Hisano et al., 2004). However, other studies have reported inconsistencies between carbohydrate accumulation and freezing tolerance (Pollock et al., 1988; Thomas and James, 1993; Maier et al., 1994; Bush et al., 2000). In our study, we found little changes in the total soluble carbohydrates (sum of glucose, fructose and

sucrose) other than at 2°C, where CB had higher crown soluble sugar content (5.22 mg · g<sup>-1</sup> DW) compared to AB (3.74 mg · g<sup>-1</sup> DW) (Figure 2.7). There were no significant changes in the soluble carbohydrates content of crowns in response to deacclimation for either species. However, in a more comprehensive investigation of leaf and crown carbohydrates, Hoffman et al. (2014b) reported a relationship between high molecular weight fructan content and the maintenance of freezing tolerance during deacclimation, with CB maintaining a higher content of HMW fructans in crowns during deacclimation at 8°C. In addition, Dionne et al. (2010) found that HMW fructan content accounted for over 50% of the variation in freezing tolerance among a collection of 42 ecotypes of AB. Therefore, specific carbohydrate fractions such as fructans seem to play a larger role compared to the accumulation of total soluble sugars in relation to cold acclimation and deacclimation capacities in these species.

In summary, the greater up-regulation of carbon metabolism of AB during deacclimation may promote faster recovery, and potentially a competitive advantage in mixed stands of CB. However, in response to mid-winter thaw events, deacclimation that may be accompanied by enhanced water uptake and metabolism of protective compounds could be detrimental, particularly if followed by subsequent freezing that would be common in field environments. Therefore, in order to reduce winter injury on golf turf, minimizing mid-winter temperature fluctuations to inhibit carbon metabolism activities is necessary, particularly where large stands of annual bluegrass exist. From management perspective, one possible solution is to utilize a covering system (ie impermeable synthetic cover or deep snow layer) during winter may be a good way to protect turf from winter injury induced by mid-winter thaw events by insulating turf from air temperature fluctuations (Dionne, 2000; Tompkins et al., 2000; Skorulski,

2002).

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Figure 2.1. Changes of freezing tolerance (lethal temperature that induces 50% kill,  $LT_{50}$ ) in annual bluegrass (AB) and creeping bentgrass (CB) following cold acclimation at 20°C, 2°C, -2°C for 2 weeks and deacclimation at 8°C for 1 day and 5 days. Bars are LSD values ( $P \leq 0.05$ ) indicating significant differences among means across species and temperature treatments.

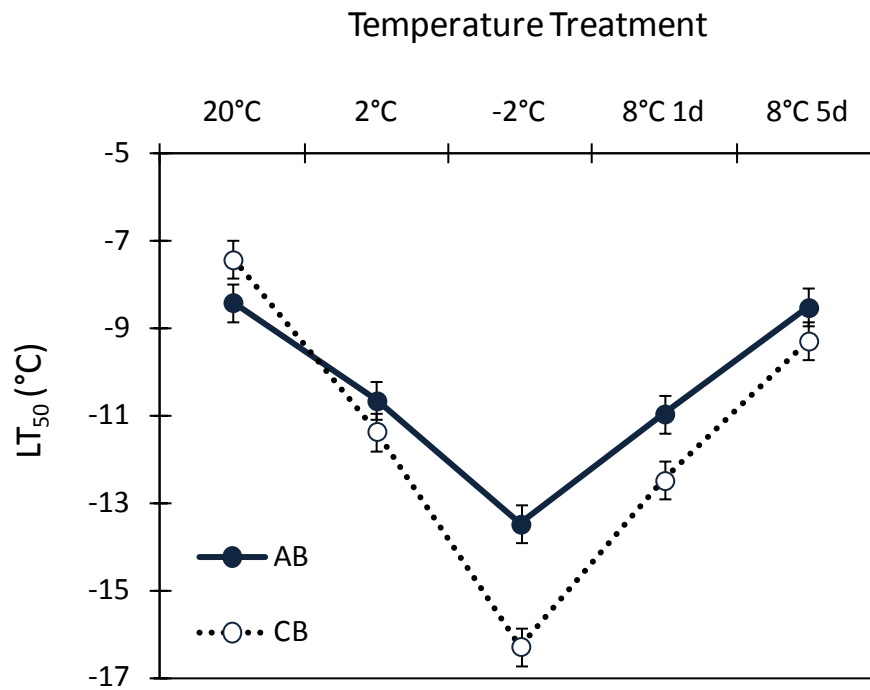


Figure 2.2. Changes of photochemical efficiency ( $F_v/F_m$ ) in annual bluegrass (AB) and creeping bentgrass (CB) following cold acclimation at 20°C, 2°C, -2°C for 2 weeks and deacclimation at 8°C for 1 day, 3 days and 5 days. Bars are LSD values ( $P \leq 0.05$ ) indicating significant differences among means across species and temperature treatments.

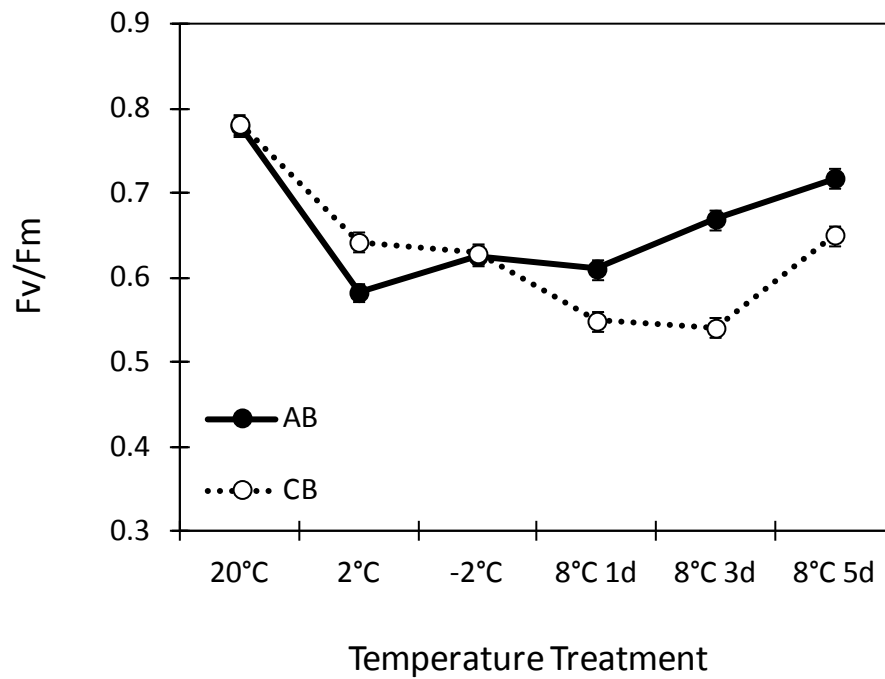


Figure 2.3. Changes of quantum yield (Yield) in annual bluegrass (AB) and creeping bentgrass (CB) following cold acclimation at 20°C, 2°C, -2°C for 2 weeks and deacclimation at 8°C for 1 day, 3 days and 5 days. Bars are LSD values ( $P \leq 0.05$ ) indicating significant differences among means across species and temperature treatments.

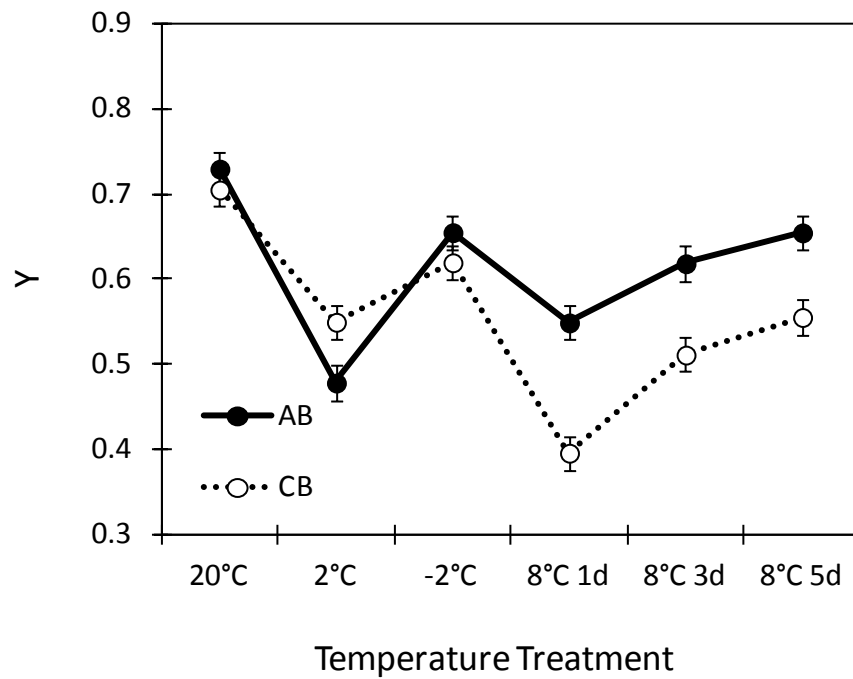


Figure 2.4. Changes of non-photochemical quenching (NPQ) in light harvesting systems of annual bluegrass (AB) and creeping bentgrass (CB) following cold acclimation at 20°C, 2°C, -2°C for 2 weeks and deacclimation at 8°C for 1 day and 5 days. Bars are LSD values ( $P \leq 0.05$ ) indicating significant differences among means across species and temperature treatments.

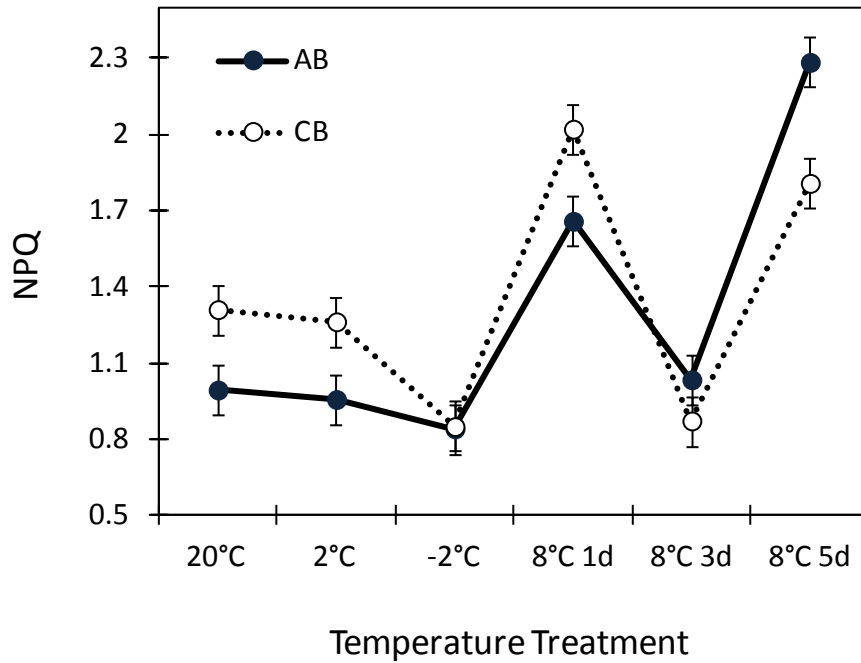


Figure 2.5. Changes of photosynthesis rate (CO<sub>2</sub> assimilation rate) in annual bluegrass (AB) and creeping bentgrass (CB) following cold acclimation at 20°C, 2°C, -2°C for 2 weeks and deacclimation at 8°C for 1 day and 5 days. Bars are LSD values (P ≤ 0.05) indicating significant differences among means across species and temperature treatments.

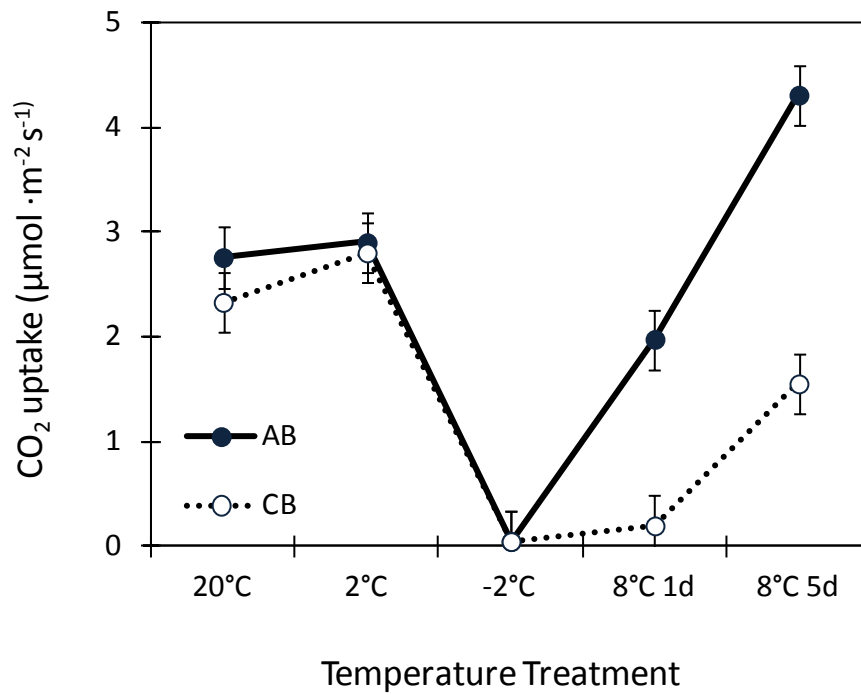


Figure 2.6. Changes of respiration rate (CO<sub>2</sub> utilization rate) in annual bluegrass (AB) and creeping bentgrass (CB) following cold acclimation at 20°C, 2°C, -2°C for 2 weeks and deacclimation at 8°C for 1 day and 5 days. Bars are LSD values (P ≤ 0.05) indicating significant differences among means across species and temperature treatments.

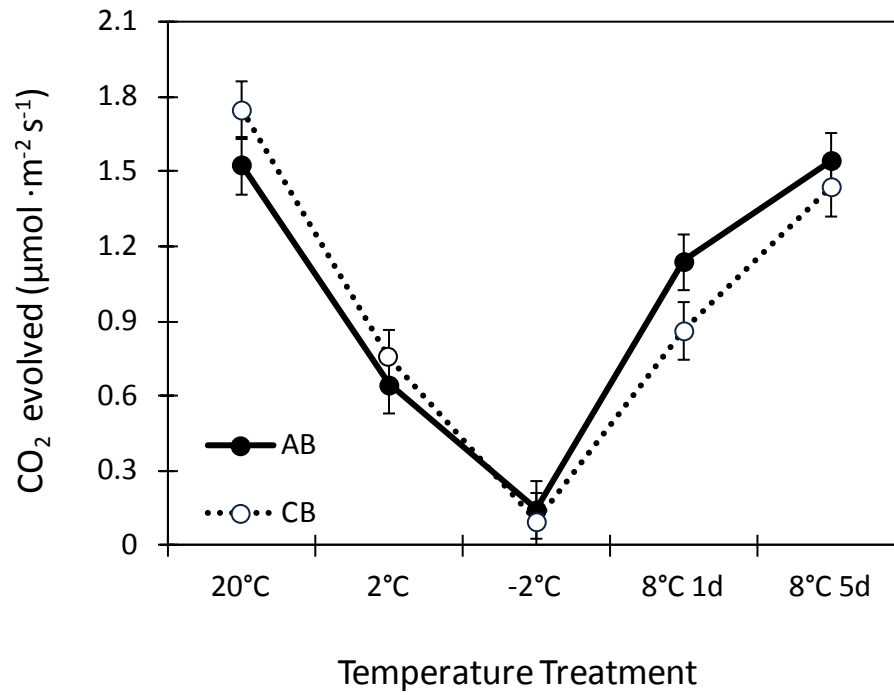


Figure 2.7. Changes of total soluble sugar contents (sum of sucrose, glucose and fructose) in annual bluegrass (AB) and creeping bentgrass (CB) following cold acclimation at 20°C, 2°C, -2°C for 2 weeks and deacclimation at 8°C for 1 day and 5 days. Bars are LSD values ( $P \leq 0.05$ ) indicating significant differences among means across species and temperature treatments.

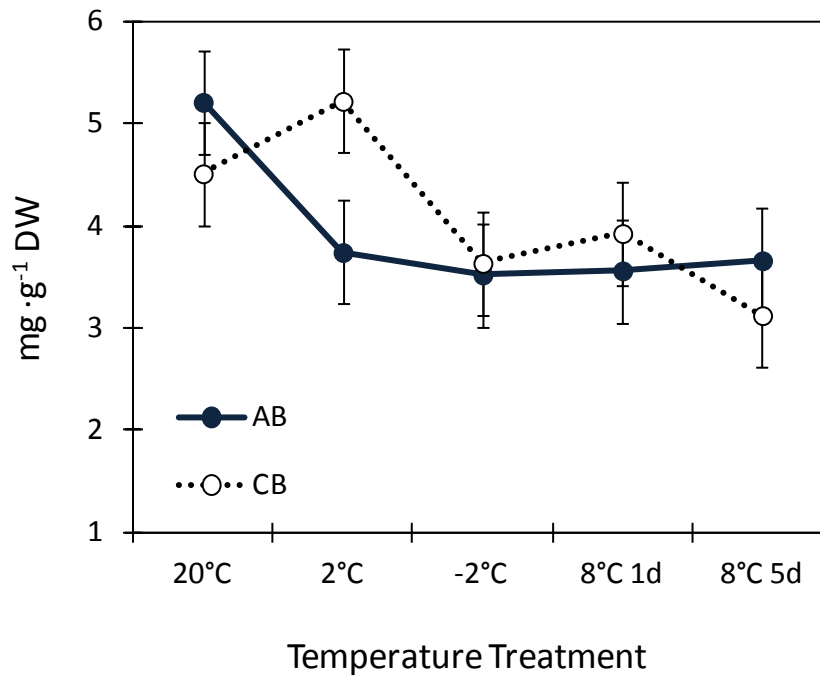




Table 2.1. Results from ANOVA of freezing tolerance (LT<sub>50</sub>), photochemical efficiency (Fv/Fm), quantum yield (Y), non-photochemical quenching (NPQ), photosynthesis (Pn), respiration (Resp) and total soluble carbohydrates (TSC) as influenced by species/ecotype (CB, AB) in response to non acclimation (20°C for 2 wk), cold acclimation (2°C for 2 wk, -2°C for 2 wk), and deacclimation (8°C 1d, 5d) treatments.

Source of variation	df	LT <sub>50</sub>	FvFm	Y	NPQ	Pn	Resp	TSC
Species	1	***	***	***	NS	***	NS	NS
Temperture treatments	4	***	***	***	***	***	***	***
Control vs. All	1	***	***	***	***	***	***	***
AC vs. DAC	1	***	NS	***	***	***	***	NS
Within AC	1	***	NS	***	***	***	***	*
Within DAC	1	***	***	***	***	***	***	NS
Species x Environment	4	***	***	***	***	***	*	NS
(AB vs. CB) x (Control vs. All)	1	***	**	***	***	*	*	NS
(AB vs. CB) x (AC vs. DAC)	1	**	***	***	***	***	NS	NS
(AB vs. CB) x (Within AC)	1	***	***	***	*	NS	NS	NS
(AB vs. CB) x (Within DAC)	1	*	***	**	***	*	NS	NS

NS, Nonsignificant

\*\*\*, \*\*, \* Significant at P ≤ 0.001, 0.01, and 0.05 probability

### CHAPTER 3

## EVALUATION OF HORMONE CHANGES IN ASSOCIATION WITH COLD ACCLIMATION AND DEACCLIMATION OF ANNUAL BLUEGRASS AND CREEPING BENTGRASS

### Abstract

Annual bluegrass (*Poa annua*) (AB) and creeping bentgrass (*Agrostis stolonifera*) (CB) are two cool-season turfgrass species that vary in their winter survival, which is associated with the intraspecific differences in cold acclimation and deacclimation capacities. In order to reduce winter injury and mitigate re-establishment costs, a better understanding of factors that contribute to freezing tolerance for these two species is necessary. Therefore, the objective of this study was to quantify changes in major hormones in leaf and crown tissues during cold acclimation and deacclimation in relation to freezing tolerance of AB and CB. Plants were propagated and established in the greenhouse for 12 months and then moved to a growth chamber for cold acclimation and deacclimation treatments, including: (1) non-acclimated control at 20 °C; (2) cold acclimated at 2 °C; (3) cold acclimated at -2 °C; (4) deacclimated at 8 °C for 1 d; and (5) deacclimated at 8 °C for 5 d. Following each treatment, changes in chlorophyll fluorescence were monitored by photochemical efficiency (Fv/Fm) and quantum yield (Y), and freezing tolerance was evaluated based on lethal temperature resulting in 50% kill (LT<sub>50</sub>). Hormones were extracted from leaves and crowns, including abscisic acid (ABA), indole-3-acetic acid (IAA), zeatin, salicylic acid (SA), and jasmonic acid (JA), using ultrafast liquid chromatography-electrospray ionization tandem mass spectrometry. Overall, CB exhibited higher freezing tolerance (lower LT<sub>50</sub>) following cold acclimation and maintained higher freezing tolerance during deacclimation, which was associated with

a lower  $F_v/F_m$  compared to AB during deacclimation. Cold acclimation was most consistently associated with increases in ABA, IAA, and SA contents of leaves and crowns. In contrast, the levels of these hormones significantly declined in response to deacclimation, in particular for AB compared to CB.

## **Introduction**

The development of freezing tolerance is an important trait required for overwintering survival of cool-season grasses, and is dependent on a cold acclimation period that is initiated on exposure to low, nonfreezing temperatures and decreases in photoperiod (Levitt, 1980). These environmental cues lead to signal transduction cascades associated with induction of cold-regulated (COR) genes, many of which result in the production of metabolites that aid in membrane stability, osmotic adjustment, and antioxidant scavenging capacity (Karpinski et al., 2002; Munshaw et al., 2006). Altogether these critical changes help to lower cellular freezing point and allow plants to withstand extensive freeze-induced cellular desiccation. Differences in the capacity to modulate the extent of these various physiological and biochemical traits during cold acclimation have been shown to contribute to species and cultivar differences in freezing tolerance (Rajashekar et al., 1983; Harrison et al., 1997; Patton et al., 2007a,b; Hoffman et al., 2010).

The occurrence of a warming event during winter or early spring can potentially reverse the cold acclimated state (i.e., cold deacclimation) and cause a reduction or complete loss of freezing tolerance. Compared to the large body of literature related to the genetics and physiology of cold acclimation, there is considerably less understood on the mechanisms regulating cold deacclimation of plants. Losses of freezing tolerance induced by deacclimation have been associated with changes such as increases in cellular water content, modifications in membrane lipid composition, and increases in the metabolism of protective solutes (Tronsmo et al., 1993; Ögren, 1997; Arora et al., 2004; Iivonen et al., 2004). If plants are exposed to elevated temperatures in mid-winter to early spring that trigger the deacclimation process, this may lead to untimely loss of freezing tolerance and low temperature kill

when temperatures subsequently drop below freezing. Consequently, the ability of plants to resist deacclimation and maintain maximum freezing tolerance may be considered an integral overwintering strategy for some plants species and account for inter- and intraspecific variations in winter survival (Eagles and Williams, 1992; Huner et al., 1998; Hoffman et al., 2014a).

Deacclimation has been reported to occur more rapidly in comparison to the time required to achieve freezing tolerance through cold acclimation, with freezing tolerance losses occurring within few days to less than two weeks upon warmer temperature exposure (Gay and Eagles, 1991; Rapacz, 2002a; Kalberer et al., 2006; Espevig et al., 2011; Hoffman et al., 2014a,b). In comparison, the development of freezing tolerance during cold acclimation may take weeks to months, depending on plant species. The extent of deacclimation can be influenced by the magnitude of temperature increase and the duration of exposure to elevated temperatures (Gay and Eagles, 1991; Eagles and Williams, 1992; Svenning et al., 1997; Kalberer et al., 2006; Gu et al. 2008; Pagter and Arora, 2013; Hoffman et al., 2014a), as well as photoperiod (Rapacz, 2002b; Junttila, 1997). Given the potential rapid losses in freezing tolerance in response to warming events, this suggests the rapid activation of signaling compounds that are involved in triggering downstream responses leading to deacclimation.

Plant hormones are an important group of signaling compounds that help regulate most aspects of plant growth and metabolism. There are five primary classes of plant hormones including abscisic acid (ABA), auxin, gibberellic acid (GA), cytokinins, and ethylene. In addition, compounds such as salicylic acid (SA), jasmonic acid (JA), and brassinosteroids have been more recently included within the plant hormone classification. Due to the widespread role of hormones, particularly in

terms of water relations, growth regulation, and triggering stress-associated metabolites, it is not surprising that some of these signaling molecules have been reported to play a role in the development of plant freezing tolerance. A majority of studies have focused on the role of ABA in freezing tolerance, where it has been shown to accumulate during cold acclimation and mediate the expression of cold regulated genes (Hajela et al., 1990; Lång and Palva, 1992; Gusta et al., 2005; Mahajan and Tuteja, 2005; Xin and Li, 1993; Semeniuk et al., 1986; Bravo et al., 1998). Other hormones associated with stimulation of cell division and growth, such as cytokinins, auxins, and GA have been shown to be down-regulated at low temperatures (Achard et al., 2008; Kosová et al., 2012). Although compounds such as SA and JA have primarily been studied in relation to pathogen or wounding responses, recent investigations have suggested relationships between the accumulation of these compounds and freezing tolerance development (Tasgín et al., 2003; Horváth et al., 2007; Janda et al., 2007; Majláth et al., 2012; Pieterse et al., 2012; Santino et al., 2013). Compared to the studies described above that focus primarily on cold acclimation responses, there is little information on how hormones are involved to regulate deacclimation of plants, particularly in relation to short and longer term warm temperature exposures.

Annual bluegrass (*Poa annua* L.) (AB) and creeping bentgrass (*Agrostis stolonifera* L.) (CB) are two cool-season grass species that vary in their winter survival. Investigations conducted under both field and controlled environmental conditions have shown that CB exhibits a greater freezing tolerance capacity compared to AB following a period of cold acclimation (Tompkins et al., 2000; Tompkins et al., 2004; Hoffman et al., 2014b). In addition, research by Hoffman et al. (2014a,b) and Guan et al. (unpublished) also demonstrated differences in the

deacclimation sensitivity of these two species, which could be observed within 1 d of exposure to warming temperatures. Therefore, the goal of the current study was to expand on our previous research and gain a more thorough understanding of the regulation of freezing tolerance in AB and CB, with a particular emphasis on the early hormone changes associated with cold deacclimation of these two species. In addition to information on fundamental processes involved in freezing tolerance of perennial grass species, a greater understanding of hormones changes involved in cold acclimation and deacclimation may also have more practical applications in turfgrass management, potentially through exogenous application of plant growth regulators to help reduce deacclimation in response to mid-winter warming events. Therefore, the specific objective of the study was to quantify changes in leaf and crown hormone concentrations at different stages of cold acclimation and deacclimation for AB and CB.

## **Materials and Methods**

### **Plant materials and growing conditions**

Plugs of one creeping bentgrass cultivar (Penncross) (collected from the University of Massachusetts Joseph Troll Turf Research Center) and one annual bluegrass biotype (collected from Longwood Cricket Club, Chestnut Hill, MA) were transplanted into containers (5 cm diameter, 25 cm depth) filled with USGA sand. Plants were maintained in greenhouse for 1 month under optimal growing conditions, which consisted of 23 °C/18 °C (day/night) temperatures, irrigated three times per week, trimmed to 1 cm height of cut and fertilized with full-strength Hoagland solution (Hoagland and Arnon, 1950) on a weekly basis. Once plants were fully established, plants were moved into a controlled environment growth chamber

(Conviron, Winnipeg, CA) and maintained at 20 °C under a 10-hour photoperiod with a photosynthesis photon flux density (PPFD) of 300  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and a relative humidity of 50%.

### **Treatments**

Plants of CB and AB were exposed to cold acclimation and deacclimation temperature environments based on the work conducted by Hoffman et al. (2014b). Temperature environments were as follows: (i) non acclimated at 20 °C for 2 weeks; (ii) cold acclimated at 2 °C for two weeks; (iii) cold acclimated at -2 °C for two weeks; (iv) deacclimated at 8 °C for 1 d; and (v) deacclimated at 8 °C for 5 d. In the growth chamber, the light level was at 300  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD for cold acclimation at 2 °C, 0  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD at -2 °C, and at 150  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD for deacclimation treatments. Following each temperature treatment, plants were harvested for freezing tolerance assessment and hormone contents.

### **Measurements**

Freezing tolerance was determined based on the lethal temperature resulting in 50% plants killed ( $LT_{50}$ ). Freezing tests were conducted following each temperature treatment to assess changes in freezing tolerance during cold acclimation and deacclimation. Ten individual plants (leaf, crown and 1 cm of roots) per replicate were wrapped in moist paper towels and placed into plastic bags for each temperature treatment according to the methods described by Ebdon et al. (2002). All bags containing plant materials were held at 2 °C for the duration of the harvest. Plants were exposed to six freezing temperatures in a programmable freezing chamber (Tenney TC Series Cycling Test Chamber, SPX Thermal Product Solutions, White



Deer, PA) as follows: -6, -9, -12, -15, -18, and -21 °C. The freezer was programmed to decrease at a rate of 2 °C per hour, along with 1 hour plateau at each freezing test temperature. After the freezing test, plants were transplanted into the cell trays containing commercial potting media (Pro-mix; Griffin Greenhouse and Nursery Supplies, Tewksbury, MA) and maintained in the greenhouse at 23/18 °C (day/night) for recovery. Following three weeks, the survival percentage at each freezing temperature was evaluated by counting the number of living plants out of the total number of plants. The  $LT_{50}$  of each species was calculated by fitting percent survival to temperature using the PROC LOGISTIC procedure.

To monitor changes in photosynthetic activity as an indirect measure of carbon metabolism, photochemical efficiency ( $F_v/F_m$ ) was measured at the end of each treatment and prior to harvest using a portable chlorophyll fluorometer (Opti-Science Inc., Hudson, NH). The fluorescence chamber was placed on the canopy of the plants at two locations per pot, and then averaged. The  $F_v/F_m$  was measured following a minimum dark adaptation period of 30 min.

At the same time as harvests for  $LT_{50}$  determination, additional leaf and crown tissues (200 mg each) were collected for hormone analyses. The tissues were washed free of soil, wrapped in foil packets, and frozen in liquid nitrogen. Following harvest, the tissues were then stored in a -80°C chamber until further analyses. Five major hormones, including ABA, auxin (IAA), cytokinin (zeatin), SA, and JA, were extracted and quantified according to the method of Liu et al. (2008) with modifications, as detailed below.

Approximately 200 mg frozen tissues (leaves and crown) were ground to a fine powder in liquid nitrogen using a mortar and pestle, and transferred to 1.5ml tubes. Ground tissues were mixed with 850 µL cold extraction buffer (methanol:

water: acetic acid, 80: 19: 1, v/v/v) and shaken vigorously at 4°C for 16 h in the dark. The tissues were then centrifuged at 14000 rpm for 20 min at 4°C, and the supernatant was transferred to a new 1.5 ml tube. The remaining pellet was re-mixed with 400 µL of the extraction buffer, shaken at 4°C for 4 h in the dark, and centrifuged at 14000 rpm for 20 min at 4°C. The supernatant from the two tubes was combined, dried using centrifugal vacuum concentrator (Labconco, MO), and then dissolved in 200 µL methanol. Approximately 100 nano moles of deuterium labelled internal standards of ABA (<sup>2</sup>H<sub>6</sub>-ABA) was added at the time of extraction. The hormones were quantified using ultra-fast liquid chromatography-electrospray ionization tandem mass spectrometry (UFLC-ESI-MS/MS) (Waters Acquity TQD, Waters, MA).

### **Experimental Design and Statistical Analyses**

The plants were completely randomized in the growth chamber. The experiment consisted of two species (AB and CB) and five temperature treatments, with 4 replicates for each species-temperature treatment combination. The data were analyzed using analysis of variance (ANOVA) according to the linear model procedure for the Statistical Analysis System v. 9.2 (SAS Institute, Inc. Cary, NC) and means were separated with Fisher's protected least significant difference (LSD) test at the 0.05 probability level. The main effects of two species, five temperature regimes, and their interaction were analyzed by partitioning the total treatment sums of squares (SS) into single degree of freedom (df) orthogonal contrasts.

### **Results and Discussion**

In response to our cold acclimation treatments (2 and -2°C), there was a significant increase in freezing tolerance (lower LT<sub>50</sub>) compared to that at 20°C for

both species (Fig. 3.1). Among the cold acclimation treatments, sub-zero acclimation at  $-2^{\circ}\text{C}$  resulted in the highest freezing tolerance, with CB achieving a significantly greater level of freezing tolerance ( $\text{LT}_{50}$  of  $-20.1^{\circ}\text{C}$ ) compared to AB ( $-14.7^{\circ}\text{C}$ ). These results are in agreement with our previous study and others comparing the cold acclimation capacity and freezing tolerance of AB and CB (Tompkins et al., 2004; Hoffman et al., 2014a,b; Guan et al., unpublished). In response to transferring plants from  $-2$  to  $8^{\circ}\text{C}$ , deacclimation was observed as early as 1 d of treatments as indicated by the significant increases in  $\text{LT}_{50}$  for both species. The freezing tolerance of AB decreased to a greater extent at  $8^{\circ}\text{C}$  for 1 d compared to CB ( $\text{LT}_{50}$  of  $-14.7$  to  $-12.0^{\circ}\text{C}$  for AB, and  $-20.1$  to  $-18.6^{\circ}\text{C}$  for CB). As plants were exposed for longer duration at  $8^{\circ}\text{C}$ , additional freezing tolerance was lost. In contrast to the species responses at  $8^{\circ}\text{C}$  for 1d, where the freezing tolerance of AB seemed to decrease at a greater rate than CB during early stages of deacclimation, deacclimation of CB was observed to be greater than AB from 1 to 5 d of deacclimation, with CB losing approximately  $4.3^{\circ}\text{C}$  ( $-18.6$  to  $-14.3^{\circ}\text{C}$ ) and AB losing approximately  $3.1^{\circ}\text{C}$  ( $-12$  to  $-8.9^{\circ}\text{C}$ ) in freezing tolerance. Hoffman et al. (2014a) recently reported that deacclimation rates for AB and CB varied depending on the deacclimation temperature ( $4$ ,  $8$ , and  $12^{\circ}\text{C}$ ) and duration (1 or 5 d), with AB exhibiting a 2.5-fold greater loss in freezing tolerance compared to CB at  $4^{\circ}\text{C}$ , whereas CB exhibiting a 3-fold greater sensitivity and loss in freezing tolerance in response to longer exposures at higher temperatures. Irrespective of rate of deacclimation, CB always exhibited a significantly lower  $\text{LT}_{50}$  compared to AB, indicating that CB maintained a higher freezing tolerance than AB during deacclimation. Following deacclimation at  $8^{\circ}\text{C}$  for 5 d, the freezing tolerance of AB was similar to the AB non-acclimated control at  $20^{\circ}\text{C}$ .

Photochemical efficiency ( $F_v/F_m$ ) was assessed as an indirect measure of the

physiological activity during cold acclimation and deacclimation. The Fv/Fm of all plants significantly decreased in response to cold acclimation at 2°C (from 0.79 to 0.68 and 0.78 to 0.64 for AB and CB, respectively) (Fig 3.2). However, contrary to the results from our previous study where AB either had lower or equal Fv/Fm to CB during cold acclimation, AB exhibited higher Fv/Fm compared to CB at both 2 and -2°C. This may be attributed to the use of different AB biotypes between the two studies, which showed variable cold acclimation capacity and freezing tolerance (data not shown). Similar results has been previously reported in a field study by Rapacz et al. (2004), in which they observed a negative correlation between Fv/Fm in autumn and winter survival in of *Festuca pratensis* × *Lolium multiflorum* hybrids, with freezing tolerant lines exhibiting a greater reduction of Fv/Fm in favor of non-photochemical quenching and protection of the photosynthesis apparatus. In response to exposure at 8°C for 1 d, the Fv/Fm of AB and CB remained at similar levels to what was observed at -2°C. By 3 and 5 d of deacclimation, Fv/Fm continued to increase, with AB always having a significantly higher Fv/Fm during deacclimation.

Plant hormones are essential molecules that signal and regulate plant growth and metabolism, and in particular play important roles in regulating plant responses to various abiotic and biotic stresses (Rikin et al. 1976; Kuiper et al., 1990; Taylor et al., 1990; Davies and Zhang, 1991; Ciardi et al. 1997; Durner et al., 1997; Mauch-Mani et al., 2005; Guo et al., 2010; Zhu et al., 2014). Plant hormones may influence plant stress responses individually, or have synergistic/antagonistic effects (Ross et al., 2000; Swarup, 2002; De Smet et al.2003; Gray, 2004; Nordström et al., 2004; Huang et al., 2014). In the current study, we quantified changes in plant hormones in relation to freezing tolerance, with particular emphasis on the hormones that could be involved in signaling cold deacclimation in response to warming temperatures. While

a majority of previous studies have focused on changes in leaf hormone levels, we performed a more comprehensive investigation on both leaf and crown tissues, which are major over-wintering structures in grass species. In general, we found that ABA, IAA, SA, and JA contents were significantly increased in crowns and/or leaves specifically in response to prolonged sub-zero acclimation at  $-2^{\circ}\text{C}$  for two weeks (Fig. 3.3, 3.4, 3.6, and 3.7). It is important to point out that sampling for hormones at 20, 2, and  $-2^{\circ}\text{C}$  occurred at the end of two weeks exposures at each temperature, and therefore may not reflect early changes in these acclimation responses (compared to our sampling during short-term deacclimation). This may help to explain why some hormones showed either no changes or decreases in certain hormone concentrations in response to  $2^{\circ}\text{C}$  cold acclimation, which is within a temperature range for cold acclimation that has previously been shown to induce significant increases in hormones such as ABA. For example, Dörffling et al. (1990) and Rapacz et al. (2003) previously reported decreases in ABA content followed a prolonged cold acclimation. More recently, a comprehensive evaluation of multiple hormones at different time points of cold acclimation (1 d, 3 to 7 d, and 21 d) showed unique hormone profiles at the different stages of acclimation, likely representing the dynamics of changes in the cell metabolism to fully adjust to low temperatures (Kosová et al., 2012). When plants were shifted from  $-2$  to  $8^{\circ}\text{C}$  to induce deacclimation, the hormones varied in their responses, which depended on species and plant tissue (ie leaves, crowns).

Both ABA and IAA contents generally declined in leaf and crown tissues in response to  $8^{\circ}\text{C}$  (Fig. 3.3 and 3.4). However, AB exhibited a greater decline in these hormones at  $8^{\circ}\text{C}$  1 d compared to CB, whereas no significant differences in crown ABA and IAA levels were detected between the two species. The relationship between ABA and freezing tolerance has been extensively studied, showing that ABA

serves as an important signaling compound that regulates gene expressions under cold stresses and contributed to freezing tolerance (Chen and Gusta, 1983; Mundy and Chua, 1988; Lång and Palva, 1992; Xin and Li, 1993; Bravo et al., 1998; Shinozaki et al., 2000). Moreover, ABA also functions in water balances to avoid cell dehydration and membrane damage (Rikin et al., 1979; Ristic and Cass, 1993), and in photosystem II protection (Rapacz, 2002a). Auxins are well known plant hormones functioning in plant development such as apical dominance, tropic responses, and root and shoot initiation (Davies, 1995). According to a study conducted by Gavelienė et al. (2013), applying auxin analogues to rapeseed (*Brassica napus*) improved the freezing tolerance due to enhanced accumulation of proline and soluble sugars. Endogenous IAA concentration was also reported by Du et al. (2012) to increase when plants were exposed to low temperature.

In comparison to ABA and IAA, leaf zeatin concentration was not significantly different among species or temperature treatments during cold acclimation at 2°C (Fig. 3.5). In the crowns, the only significant change was the increase in AB zeatin content at -2°C. During deacclimation, zeatin content of CB leaves significantly increased at 8°C 1 d, and CB maintained higher levels of zeatin during deacclimation (1 and 5 d) compared to AB. In fact, based on single-degree of freedom orthogonal contrasts, the leaf zeatin content was significantly higher for cold deacclimation treatments compared to cold acclimation treatments, regardless of species. The increased concentration of zeatin in leaves during deacclimation may result in the up-regulation of photosynthesis-related genes (Boonman et al., 2007; Zubo et al., 2008), as well as increase in growth (as related to cytokinin-induced cell division) (Xia et al., 2009). In a study using transgenic tall fescue (*Festuca arundinaceae* Shreb.) transformed with the *ipt* gene (isopentenyltransferase),

enhanced cytokinin biosynthesis at low temperature was associated with enhanced chlorophyll content and tillering capacity (Hu et al., 2005).

Salicylic acid is a plant hormone that has mostly been studied in relation to pathogen responses (Farmer and Ryan 1992, Jackson and Taylor 1996; Mou et al., 2003). In response to low temperature stress, SA was found to be partially involved in growth inhibition (Scott et al., 2004). Pocięcha et al. (2009) also reported that freezing tolerant *Festulolium* genotypes were characterized by less accumulation of SA (and higher ABA) compared to freezing sensitive genotypes. In our study, there was a significant increase in leaf SA content in response to -2°C acclimation, but no species differences were detected (Fig. 3.6). In crowns, only AB exhibited higher SA content at -2°C. In response to deacclimation at 8°C 1d, no significant changes in leaf SA were detected. However, crown SA content for AB increased significantly at 8°C 1d and then declined by 5 d, whereas no changes were found for CB in response to cold acclimation or deacclimation.

The focus of JA research has centered on plant pathogen resistance and wounding responses (Pieterse et al., 2012), whereas less is understood regarding the role of JA in abiotic stress tolerance. Under both abiotic and biotic stresses, JA was found to participate in stress signaling (Santino et al., 2013), and regulate gene expression (Tuteja, 2009). Studies also found that JA could cross talk with other hormones such as ABA (Ton and Mauch-Mani, 2004), GA (Wasternack and Hause, 2013), and SA (Ferrari et al., 2003) within stress signaling pathways. In response to low temperature, JA was found to contribute to chilling tolerance, which was associated with its role in modifying cell membranes at low temperature (Santino et al., 2013). Its accumulation has also been associated with increased resistance to snow mold pathogens (Gaudet et al., 2011), which would be an important trait for grasses

such as CB and AB. In general, the leaf JA content did not vary based on cold acclimation treatments for both AB and CB (Fig. 3.7). In crowns, there was a significant increase in JA content following -2°C acclimation. However, following exposure at 8°C for 1 d, leaf JA level in CB increased significantly, whereas no changes were observed for AB. In general, CB had higher leaf and crown JA contents, regardless of temperature treatment.

In summary, CB exhibited higher freezing tolerance (lower LT<sub>50</sub>) following cold acclimation and maintained higher freezing tolerance during deacclimation, which was associated with a lower Fv/Fm compared to AB during deacclimation. Cold acclimation was most consistently associated with increases in ABA, IAA, and SA contents of leaves and crowns. In contrast, the levels of ABA and IAA declined more rapidly in leaves of AB compared to CB during deacclimation. The zeatin,(leaf) and JA (leaf and crown) contents of CB were generally higher than levels found in AB regardless of temperature treatments, and in particular under deacclimation treatments. In contrast to these responses, the SA content of crowns was significantly greater for AB compared to CB. Additional research is necessary to also integrate information on the changes in GA content, which could not be completed in the current study due to limitation of plant tissues.

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Figure 3.1. Freezing tolerance ( $LT_{50}$ ) of annual bluegrass (AB) and creeping bentgrass (CB) in response to cold acclimation (2, -2°C) and deacclimation (8°C for 1 and 5 d). Vertical bars are LSD values ( $p \leq 0.05$ ) representing statistically significant differences across species and different temperature treatments.

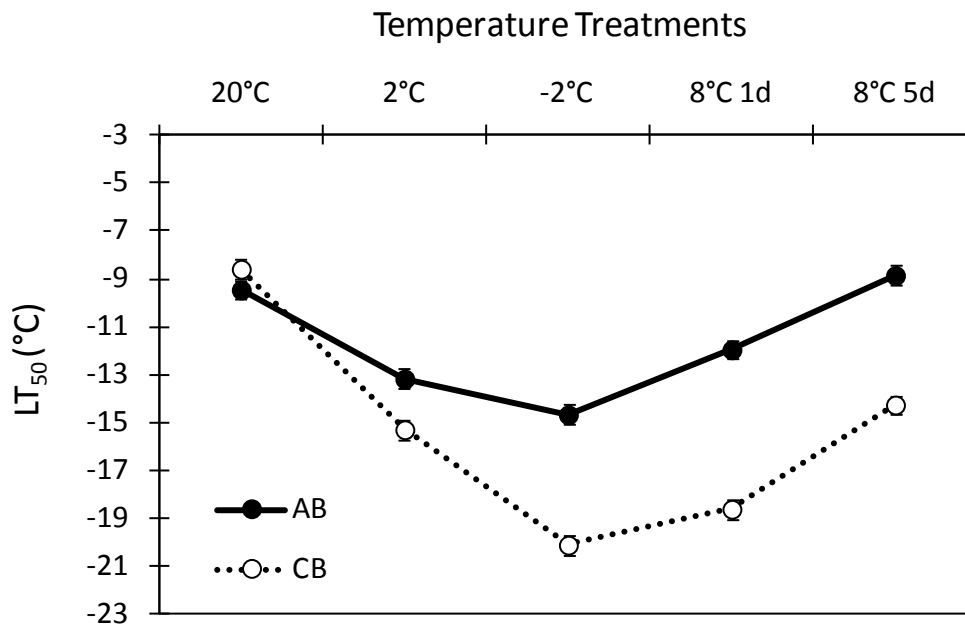




Figure 3.2. Photochemical efficiency ( $F_v/F_m$ ) of annual bluegrass (AB) and creeping bentgrass (CB) in response to cold acclimation (2, -2°C) and deacclimation (8°C for 1 and 5 d). Vertical bars are LSD values ( $p \leq 0.05$ ) representing statistically significant differences across species and different temperature treatments.

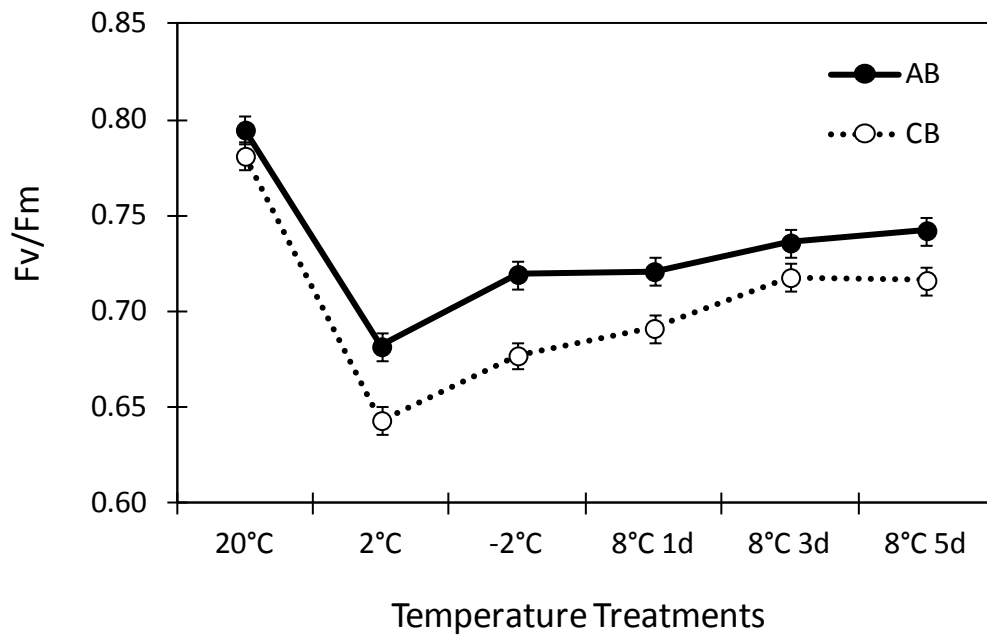


Figure 3.3. Changes in leaf (A) and crown (B) abscisic acid (ABA) concentration (ng/g FW) of annual bluegrass (AB) and creeping bentgrass (CB) in response to cold acclimation (2, -2°C) and deacclimation (8°C for 1 and 5 d). Vertical bars are LSD values ( $p \leq 0.05$ ) representing statistically significant differences across species and different temperature treatments.

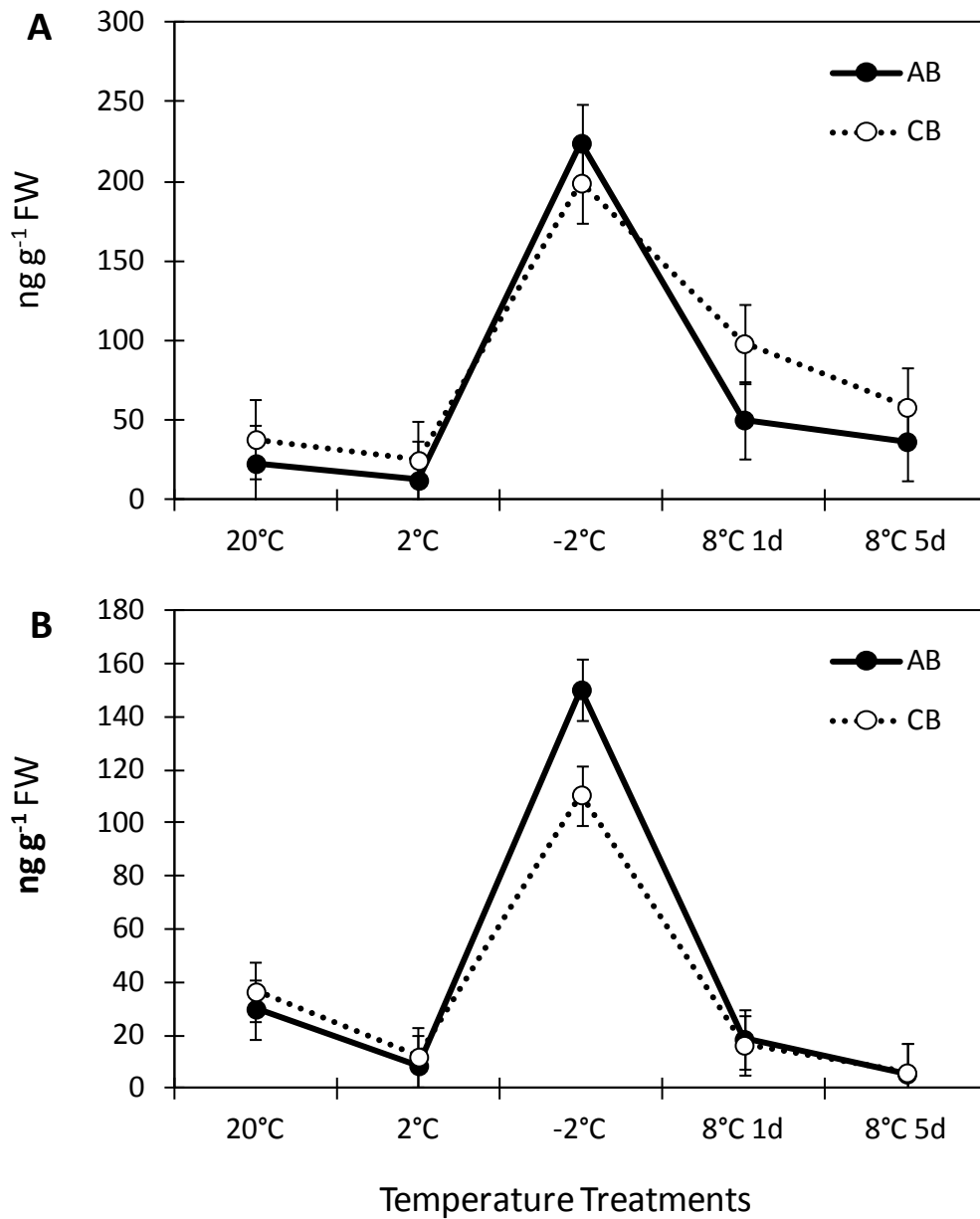


Figure 3.4. Changes in leaf (A) and crown (B) auxin (IAA) concentration (ng/g FW) of annual bluegrass (AB) and creeping bentgrass (CB) in response to cold acclimation (2, -2°C) and deacclimation (8°C for 1 and 5 d). Vertical bars are LSD values ( $p \leq 0.05$ ) representing statistically significant differences across species and different temperature treatments.

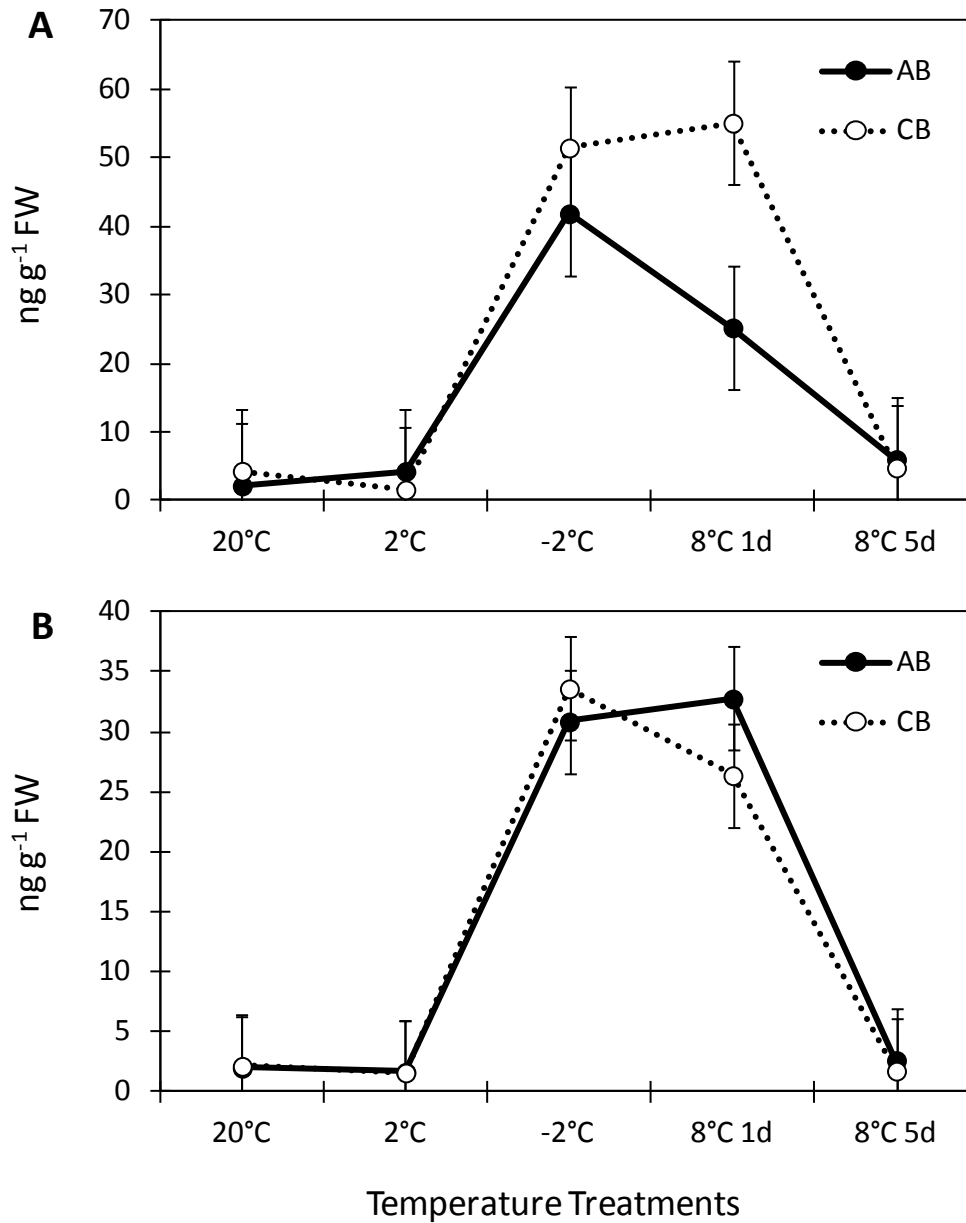


Figure 3.5. Changes in leaf (A) and crown (B) cytokinins (zeatin) concentration (ng/g FW) of annual bluegrass (AB) and creeping bentgrass (CB) in response to cold acclimation (2, -2°C) and deacclimation (8°C for 1 and 5 d). Vertical bars are LSD values ( $p \leq 0.05$ ) representing statistically significant differences across species and different temperature treatments.

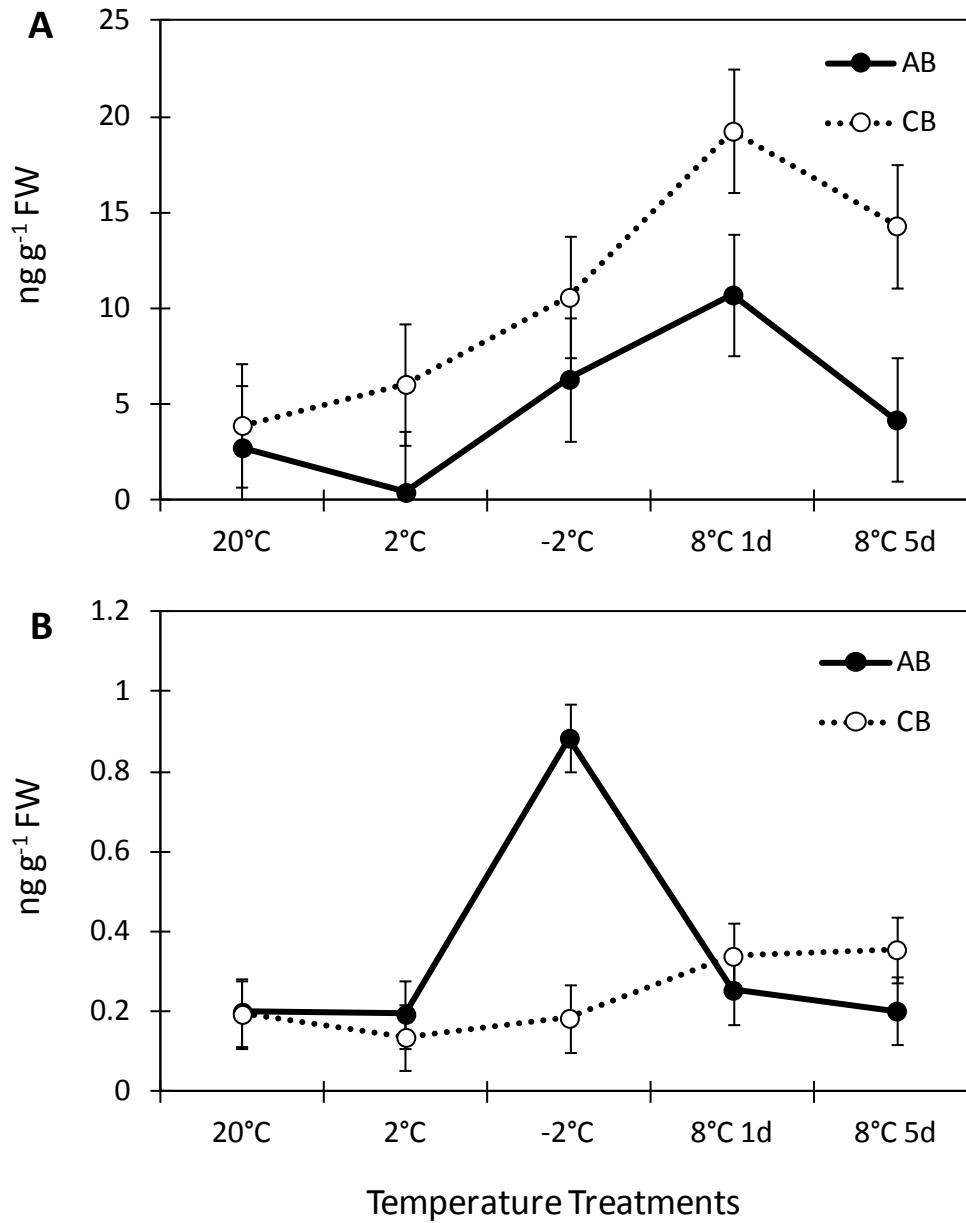


Figure 3.6. Changes in leaf (A) and crown (B) salicylic acid concentration (ng/g FW) of annual bluegrass (AB) and creeping bentgrass (CB) in response to cold acclimation (2, -2°C) and deacclimation (8°C for 1 and 5 d). Vertical bars are LSD values ( $p \leq 0.05$ ) representing statistically significant differences across species and different temperature treatments.

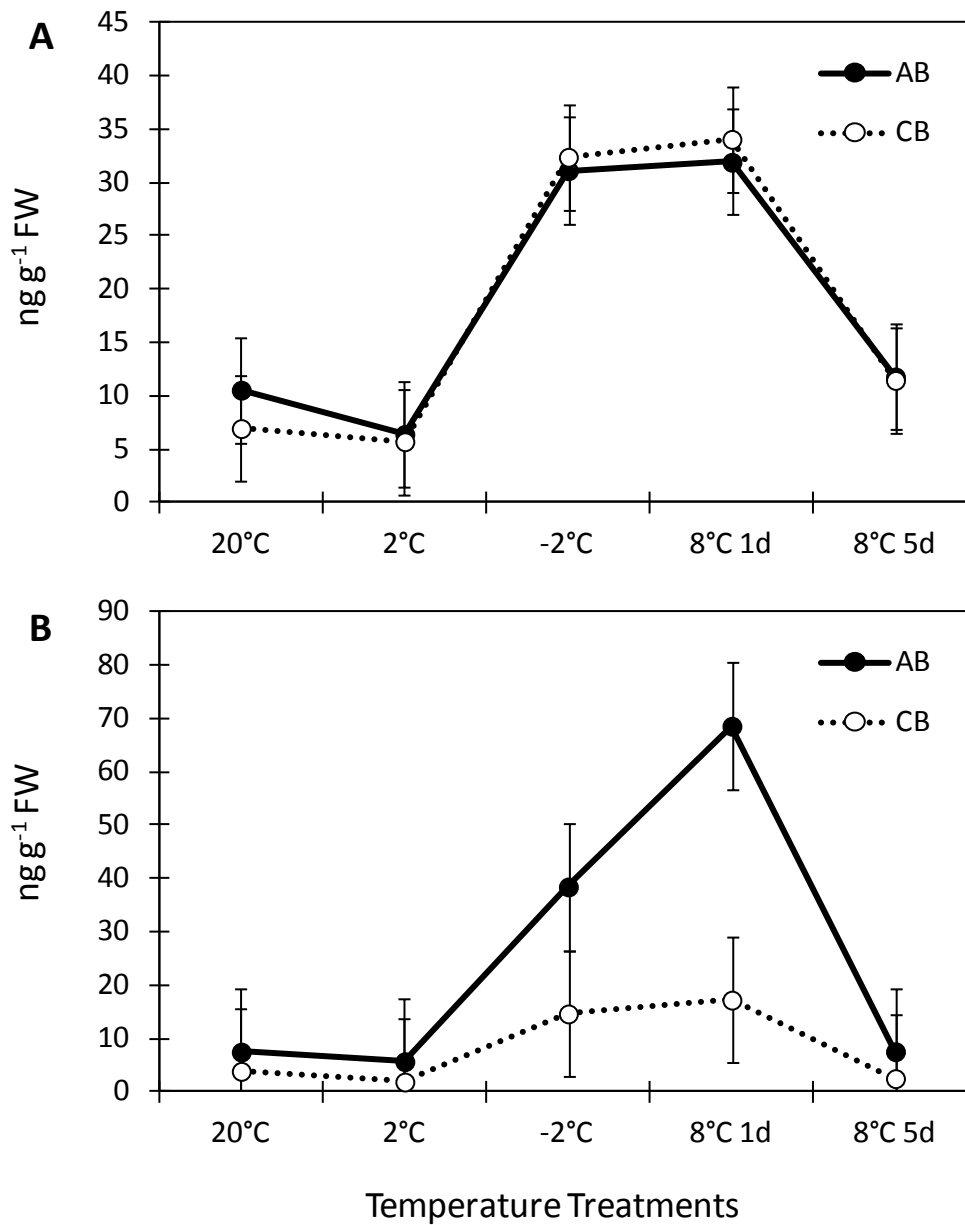


Figure 3.7. Changes in leaf (A) and crown (B) jasmonic acid concentration (ng/g FW) of annual bluegrass (AB) and creeping bentgrass (CB) in response to cold acclimation (2, -2°C) and deacclimation (8°C for 1 and 5 d). Vertical bars are LSD values ( $p \leq 0.05$ ) representing statistically significant differences across species and different temperature treatments.

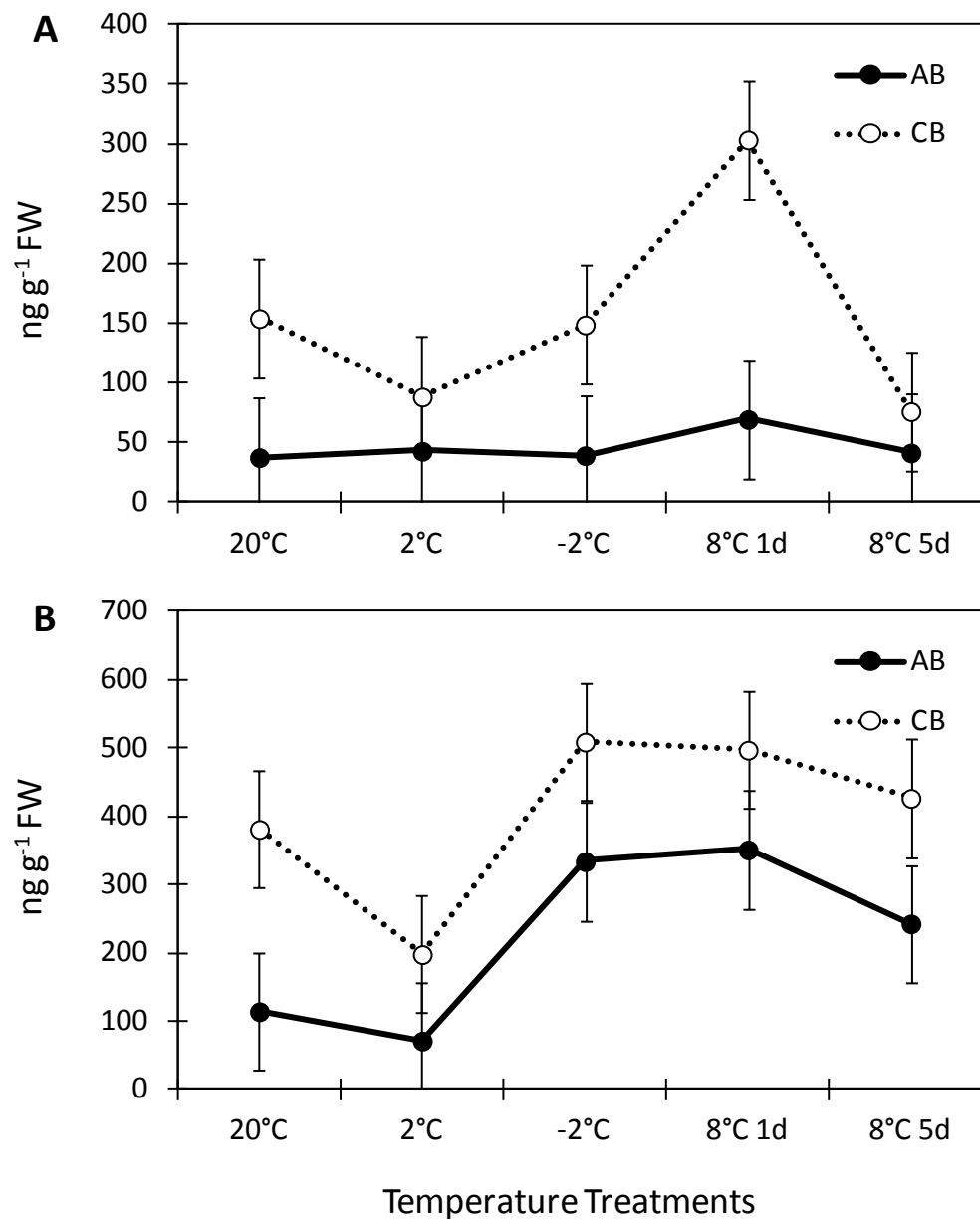


Table 3.1. Results from ANOVA for freezing tolerance (LT<sub>50</sub>) and photochemical efficiency (Fv/Fm) for two species (annual bluegrass and creeping bentgrass) and five temperature environments including non-acclimation (20°C for 2 wk), cold acclimation (2°C for 2 wk, -2°C for 2 wk), and deacclimation (8°C 1d, 5d) treatments.

Source of variation	df	LT <sub>50</sub>	Fv/Fm
Species	1	***	***
Environment	4	***	***
Control vs. All	1	***	***
AC vs. DAC	1	***	***
Within AC	1	***	***
Within DAC	1	***	***
Species x Environment	4	***	*
(AB vs. CB) x (Control vs. All)	1	***	*
(AB vs. CB) x (AC vs. DAC)	1	***	***
(AB vs. CB) x (Within AC)	1	***	NS
(AB vs. CB) x (Within DAC)	1	**	NS

NS, Nonsignificant

\*\*\*, \*\*, \* Significant at P ≤ 0.001, 0.01, and 0.05 probability

Table 3.2. Results from ANOVA of Leaf hormone contents (SA, JA, ABA, IAA, CK) ( $\text{ng g}^{-1}$ ) as influenced by species/ecotype (CB, AB) in response to non-acclimation ( $20^{\circ}\text{C}$  for 2 wk), cold acclimation ( $2^{\circ}\text{C}$  for 2 wk,  $-2^{\circ}\text{C}$  for 2 wk), and deacclimation ( $8^{\circ}\text{C}$  1d, 5d) treatments.

Source of variation	df	Leaf Hormone Content				
		SA	JA	ABA	IAA	CK
Species	1	NS	***	NS	NS	***
Environment	4	***	***	***	***	***
Control vs. All	1	***	Ns	***	***	**
AC vs. DAC	1	NS	**	***	NS	***
Within AC	1	***	NS	***	***	*
Within DAC	1	***	***	NS	***	*
Species x Environment	4	NS	***	NS	NS	NS
(AB vs. CB) x (Control vs. All)	1	NS	NS	NS	NS	NS
(AB vs. CB) x (AC vs. DAC)	1	NS	*	NS	NS	NS
(AB vs. CB) x (Within AC)	1	NS	NS	NS	NS	NS
(AB vs. CB) x (Within DAC)	1	NS	***	NS	NS	NS

NS, Nonsignificant

\*\*\*, \*\*, \* Significant at  $P \leq 0.001$ , 0.01, and 0.05 probability.



Table 3.3. Results from ANOVA of Crown hormone contents (SA, JA, ABA, IAA, CK) (ng g<sup>-1</sup>) as influenced by species/ecotype (CB, AB) in response to non-acclimation (20°C for 2 wk), cold acclimation (2°C for 2 wk, -2°C for 2 wk), and deacclimation (8°C 1d, 5d) treatments.

Source of variation	df	Crown Hormone Content				
		SA	JA	ABA	IAA	CK
Species	1	**	***	NS	NS	**
Environment	4	***	***	***	***	***
Control vs. All	1	*	NS	NS	***	*
AC vs. DAC	1	NS	*	***	NS	NS
Within AC	1	**	***	***	***	***
Within DAC	1	***	NS	NS	***	NS
Species x Environment	4	*	NS	*	NS	***
(AB vs. CB) x (Control vs. All)	1	NS	NS	NS	NS	NS
(AB vs. CB) x (AC vs. DAC)	1	NS	NS	NS	NS	***
(AB vs. CB) x (Within AC)	1	NS	NS	*	NS	***
(AB vs. CB) x (Within DAC)	1	**	NS	NS	NS	NS

NS, Nonsignificant

\*\*\*, \*\*, \* Significant at  $P \leq 0.001$ , 0.01, and 0.05 probability.

**CHAPTER 4**

**IDENTIFYING THE PHYSIOLOGICAL CHANGES ASSOCIATED WITH  
COLD ACCLIMATION AND DEACCLIMATION IN PERENNIAL  
RYEGRASS GENOTYPES WITH CONTRASTING FREEZING  
TOLERANCE**

**Abstract**

Perennial ryegrass (PR) is an economically important turf and forage grass species, but often exhibits poor winter survival. A better understanding of the mechanisms required for freezing tolerance and overwintering capacity, from genetic to whole-plant physiology, is required in order to select and breed for better adapted cultivars of PR. Therefore, we conducted initial screening tests of eight PR breeding accessions and selected two: one freezing tolerant (T73), and one freezing sensitive (S16). Plants were exposed to five temperature treatments including: (1) non-acclimated control at 20°C for 2 week, (2) cold acclimated 2°C for 2 weeks, (3) cold acclimated -2°C for 2 weeks, (4) deacclimated at 4°C for 1d, (5) deacclimated at 4°C for 5 d. During each treatment, plants were harvested and exposed to freeze tests for freezing tolerance ( $LT_{50}$ ) evaluation. Chlorophyll fluorescence measurements were quantified as an indirect measure of carbon metabolism, including: photochemical efficiency ( $F_v/F_m$ ), quantum yield (Y), and non-photochemical quenching (NPQ). In addition, crown moisture content, leaf growth rate (LGR) and tiller numbers were assessed. Our results indicated that T73 exhibited significantly lower  $LT_{50}$  than S16 during cold acclimation and deacclimation, indicating a higher freezing tolerance. In addition, T73 exhibited significantly higher  $F_v/F_m$  and Y following acclimation and deacclimation, indicating higher photosynthetic efficiency. The crown moisture content of S16 was significantly higher compared to T73 in response to 2°C

acclimation and 4°C 1d deacclimation, which may contribute to higher freezing injury potential. The LGR and relative tiller number were significantly reduced following 2 and -2°C acclimation, but increased in response to deacclimation. However, no genotype differences in LGR or relative tiller number were observed.

## **Introduction**

Perennial ryegrass (*Lolium perenne* L.) (PR) is one of the most widely utilized temperate grass species selected for turf and forage uses, along with more recent interests for this species as a producer of biomass for conversion to biofuel (Thorogood, 2003, El Bassam, 2010). This species is generally known for excellent turf quality characteristics related to color and density, as well as rapid establishment and superior traffic tolerance. As a result, PR is widely used as a species of choice alone or in mixtures for turf stands on home lawns, athletic fields, and golf courses. However, among cool-season grasses, PR is sensitive to temperature extremes, particularly freezing temperatures. For example, killing temperatures have been observed as high as -5 to -15 °C in comparison to other cool-season turfgrasses (Beard, 1973; Gusta et al., 1980), which makes it highly susceptible to winter injuries (Taylor et al., 1997). Previous research also found that freezing tolerance can significantly vary among different cultivars or genotypes within PR (Ebdon et al., 2002; Hulke et al., 2007; Hoffman et al., 2010). Therefore, improved freezing tolerance and winter hardiness traits are important breeding goals for this species (Thorogood, 2003).

Earlier research has focused on traits related to the capacity of PR plants to acclimate to freezing temperatures (Hoffman et al., 2010). During the process of cold acclimation, a series of physiological and biochemical changes within plants prepare cells to withstand extensive periods of ice-induced desiccation, including the accumulation of non-structural carbohydrates (Koster and Lynch, 1992; Dionne et al., 2010; Ball et al., 2002; Patton et al., 2007a; Espevig et al., 2011) and proline (Dörffling et al., 1997; Dionne et al., 2001b; Patton et al., 2007a; Hoffman et al., 2010), induction of cold regulated proteins (Patton et al., 2007b; Zhang et al., 2009;

Zhang et al., 2011), and alterations in cell membrane lipids and composition (Lynch and Steponkus, 1987; Samala et al., 1998; Cyril et al., 2002; Munshaw, 2004; Hoffman et al., 2010). The capacity to adjust cell metabolism during cold acclimation to maximize cell freezing tolerance depends on several factors, including plant genotype, environmental conditions, as well as general agronomic practices (Webster and Ebdon, 2005; Hulke et al., 2007; Hulke et al., 2008; Hoffman et al., 2010).

In contrast to studies on cold acclimation of PR, there have been few examinations on the deacclimation sensitivity of this species (Gay and Eagles, 1991; Eagles and Williams, 1992). The potential for deacclimation and winter damage can be affected by the magnitude of temperature increase and the duration of exposure to elevated temperatures (Gay and Eagles, 1991; Eagles and Williams, 1992; Svenning et al., 1997; Kalberer et al., 2006; Gu et al. 2008; Patgers and Arora, 2013; Hoffman et al., 2014a). Although temperature seems to be the primary factor triggering deacclimation (Patger and Arora, 2013), increases in photoperiod can exacerbate the deacclimation response (Eagles, 1994; Junttila, 1997; Rapacz, 2002). The extent of deacclimation and reacclimation capacity in response to mid-winter thawing events seems to be associated with whole-plant growth responses, such that deacclimation becomes irreversible under conditions where the plant initiates regrowth and water uptake (Rapacz et al., 2001; Rapacz, 2002; Arora et al., 2004). Previous research conducted by Webster and Ebdon (2005) reported a strong correlation between shoot growth rate, crown moisture content, and freezing tolerance ( $LT_{50}$ ) in perennial ryegrass, where higher shoot growth rates were associated with lower freezing tolerance as crown hydration increased with shoot growth.

From our previous studies using creeping bentgrass (*Agrostis stolonifera* L.) and annual bluegrass (*Poa annua* L.), we determined that increased carbon

metabolism and changes in leaf and crown hormones were associated with the losses of freezing tolerance in response to short-term shifts in temperatures (Hoffman et al., 2014b; Guan et al., unpublished). To better understand these and other mechanisms contributing to differences in deacclimation sensitivity among plants, we selected to look more closely at PR, a species for which there is greater relative genetic information compared to creeping bentgrass and annual bluegrass. For example, genetic linkage maps for PR have been developed that are aligned with maps of other *Poaceae* species (Jones et al., 2002a,b; Inoue et al., 2004; Warnke et al., 2004; Sim et al., 2005), which could facilitate comparative studies for the identification of genes important for PR winter hardiness. Therefore, the goal of the current study was to establish baseline physiological information related to cold acclimation and deacclimation traits in PR, using genotypes that vary in their freezing tolerance capacity. Specifically, the objectives of the study were to (i) evaluate freezing tolerance of eight breeding accessions of PR and select a freezing tolerant and freezing sensitive genotype; and (ii) examine how changes in photosynthetic parameters, crown hydration, leaf growth rates, and relative tiller numbers are related to differences in cold acclimation and deacclimation capacities among the two genotypes.

## **Materials and Methods**

### **Experiment 1**

Eight accessions were obtained from the University of Minnesota perennial ryegrass breeding program based on differences in winter survival, designated as: TOL73, TOL74, TOL88, TOL89 (representing tolerant accessions) and SUS08, SUS16, SUS23, SUS38 (representing susceptible accessions) (Erik Watkins, personal

communication). All plant materials were seeded in May 2012, and cultivated in the green house for five months before transplanting into cell trays and moving into the growth chamber.

For cold acclimation and deacclimation regimes, plants were exposed to four temperature treatments including: (i) non-acclimated at 20 °C for 2 weeks; (ii) cold-acclimated at 2 °C for 2 weeks; (iii) cold-acclimated at -2°C for 2 weeks; (iv) deacclimated at 8 °C for 3 d. At each treatment, the relative humidity level in the growth chamber was held at 50%, and the photoperiod was set at 10 hours/day. The light level was 320 $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD for non acclimation and 2°C cold acclimation treatments, and 0  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD for -2°C. For deacclimation treatments, the light level was adjusted to 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$  to avoid photooxidation conditions.

Freezing tolerance was determined based on the value of lethal temperature that induces 50% plant killed (LT<sub>50</sub>). Plant materials were harvested at the end of each treatment to assess freezing tolerance of the plant using the methods previously described by Ebdon et al. (2002). Plants were harvested from soil and separated into individual tillers with leaf, crown and 1 cm of roots attached. In order to induce the ice nucleation, 10 tillers of one plant genotype was wrapped into a moist paper towel and placed into a plastic bag. A total of 4 replicates (40 tillers) were placed in one plastic bag for each freezing temperature test. All bags containing plant materials were held at 4 °C for until the harvest was completely finished. Plants were exposed to six freezing temperatures including -6, -9, -12, -15, -18, and -21 °C in a programmable freezing chamber (Tenney TC Series Cycling Test Chamber, SPX Thermal Product Solutions, White Deer, PA). Temperature in the freeze chamber was programmed to decrease at a rate of 2 °C per hour. In addition, a soaking period for 1 hour was programmed when temperature in the chamber reached the freezing

temperatures as listed above. After the freeze tests, plant tillers were replanted into the cell trays containing commercial potting media (Pro-mix; Griffin Greenhouse and Nursery Supplies, Tewksbury, MA) and moved to the greenhouse at 23/18 °C (day/night) for recovery. Water was supplied as needed and full strength Hoagland's solution (Hoagland and Arnon, 1950) was applied once per week. Three weeks later, plant survival percentage at each freezing temperature was evaluated by counting the number of living plants out of the total number of plants. The  $LT_{50}$  of each genotype was calculated by fitting percent survival to temperature using the PROC LOGISTIC procedure.

## **Experiment 2**

Based on variations in freezing tolerance from Experiment 1, two PR genotypes were propagated from previous accessions were selected, including T73 (designated as freezing tolerant) and S16 (designated as freezing susceptible). Plants were vegetatively propagated into 10.16 cm square pots and maintained in the greenhouse at 23 °C/18 °C (day/night) temperatures. During the time period, plants were irrigated 3 times per week, trimmed to approximately 6 cm height of cut and fertilized with full strength Hoagland solution (Hoagland and Arnon, 1950) on a weekly basis. Eight months later, plants were moved to a controlled environment growth chamber (Convicon, Winnipeg, CA) where plants were exposed to five temperature treatments as described in Experiment 1 (20, 2, -2 °C to induce cold acclimation and 4 °C to induce deacclimation).

Following each temperature treatment, plants were harvested for freezing tolerance based on the value of lethal temperature that resulted in 50% plant mortality ( $LT_{50}$ ) as described above. Additional crown tissues were harvested to assess changes



in crown moisture content. Approximately 300 mg of crown tissues (leaves and roots removed) were harvested and immediately weighed to record fresh weight (FW). Crowns were then wrapped in aluminum foil packets and placed in an oven at 70°C for a minimum of 72 h prior to measuring dry weight (DW). Crown moisture content was calculated as:  $(FW-DW)/FW*100\%$ .

Plant growth changes including leaf elongation rate and tiller number were monitored using a second subset of plants grown in cell trays. One tray containing 10 individual tillers (2 to 3 tillers per cell) of each genotype was prepared in the greenhouse 3 weeks before the start of the treatments, indicating that each genotype had 10 replications for this assessment. During each treatment, leaf elongation rate was measured using a ruler. The grass leaf was straightened on the ruler from the bottom of the crown to the tip of the leaf. The number of tillers was measured by counting non-senescent tillers in each cell. Data were normalized based to the number of tillers per cell under non-acclimating conditions (20°C).

Chlorophyll fluorescence parameters including quantum yield (Y), photochemical efficiency (Fv/Fm), and non-photochemical quenching (NPQ) were measured with a portable chlorophyll fluorometer (Opti-Science Inc., Hudson, NH). The fluorescence chamber was placed on the plant canopy at two locations per pot, and the data was averaged over two measurements. To be specific, Fv/Fm was measured following a 30 min dark adaptation period and calculated as  $(Fm-F_0)/Fm$ , representing the ratio of variable fluorescence to maximum fluorescence. The  $F_0$  refers to the minimal fluorescence level measured by applying the modulated weak beam on the canopy while Fm refers to the maximum fluorescence level under saturated beam. The other two fluorescence measurements (Y and NPQ) were conducted under the ambient light levels, and NPQ was calculated based on the following formulas:  $NPQ=$

$(F_m - F_{ms})/F_{ms}$ , where  $F_{ms}$  refers to the maximum fluorescence in a steady fluorescence condition.

### **Experimental Design and Statistical Analyses**

For both Experiment 1 and Experiment 2, plants were completely randomized in the growth chamber. A total of four (for Experiment 1) and five (for Experiment 2) temperature treatments with 4 replicates for each genotype were designed in the study. The data were analyzed using analysis of variance (ANOVA) with SAS v. 9.4. (SAS Institute, Inc.) and the means were separated with Fisher's protected least significant difference (LSD) test at the 5% probability level. In Experiment 2, the main effects of genotypes, temperature regimes, and their interaction were analyzed by partitioning the total treatment sums of squares (SS) into single degree of freedom (df) orthogonal contrasts. Contrasts for genotypes included the main effect of T73 versus S16, while temperature regime included four orthogonal linear contrasts for comparing (i) mean of non-acclimated versus the combined mean for all acclimation and deacclimation treatments; (ii) mean of acclimation at 2 and -2 °C versus the combined mean for deacclimation at 4 °C for 1 and 5 d; (iii) mean of acclimation at 2 versus -2 °C; and (iv) mean for deacclimation at 4°C for 1 versus 5 d. Species and temperature regime main effects and associated contrasts were crossed to partition species × temperature regime interaction SS to test for single df interaction components.

### **Results and Discussion**

In Experiment 1, which served as our preliminary screening tests, we evaluated the freezing tolerance changes of eight PR accessions previously shown to exhibit differences in their overwintering capacity in Minnesota. We found that under

non-cold acclimated conditions at 20°C for 2 weeks, there were no significant differences in freezing tolerance (LT<sub>50</sub> ranging from -9.20°C to -9.40°C) (Table 4.1). Following cold acclimation at 2°C for 2 weeks, the LT<sub>50</sub> of the eight genotypes all decreased 2-3°C, indicating an increase in freezing tolerance. However, there was no significant difference detected in freezing tolerance among the accessions (LT<sub>50</sub> ranged from -11.3°C to -12.2°C). Following 2 weeks of -2°C cold acclimation, S16 and S08 had the lowest freezing tolerance (LT<sub>50</sub> of -17.9 and -18.1°C respectively), whereas T73 and S38 exhibited the highest freezing tolerance (-21.9°C and -21.8°C, respectively). After being deacclimated at 8°C for 3 days, the increased to similar levels to that observed at 20°C. However, there were no significant differences observed between the accessions. Based on these results, we selected T73 and S16 to serve as freezing tolerant and freezing susceptible genotypes, respectively, for Experiment 2.

As described earlier, one of our objectives were to quantify major changes in photosynthetic efficiency of PR, since photosynthesis adaptation in response to low temperature has been reported to be able to influence cold acclimation process (Pocock et al., 2001). In addition, changes in crown moisture content, leaf growth rates and number of tillers (as related to senescence or generation of new tillers) in response to cold acclimation and deacclimation were also investigated as these traits have been previously associated with deacclimation sensitivity and freezing injury (Fowler and Gusta, 1977; Leinonen et al., 1997; Webster and Ebdon, 2005; Tompkins et al., 2000; Rapacz, 2002).

Similar to our pre-tests in Experiment 1, we confirmed that T73 exhibited better overall freezing tolerance following cold acclimation compared to S16 (Fig. 4.1). One difference was that following 2 weeks of 2°C acclimation, where T73

exhibited higher freezing tolerance than S16, but these differences were not detected in Experiment 1. In response to deacclimation, after being exposed at 4°C for 1 d, the freezing tolerance of S16 was reduced, whereas no significant changes in freezing tolerance were detected for T73, suggesting that S16 had a higher deacclimation sensitivity to temperature increase compared to T73. Following 5 days of 4°C deacclimation, S16 did not present significant changes in freezing tolerance, but freezing tolerance of T73 was reduced.

Crown moisture content (CMC) is one of the major factors associated with freezing injury during deacclimation, including turfgrasses. Crown hydration was negatively related to freezing tolerance for perennial ryegrass (Webster and Ebdon, 2005), as well as in annual bluegrass and creeping bentgrass (Tompkins et al., 2000). Generally, when temperature drops below the freezing point, a high percentage of crown hydration can result in intracellular ice formation, which will ultimately result in cell death. In the current study, the CMC of both genotypes decreased significantly during cold acclimation at 2 and -2°C (Fig. 4.2). It seemed that the reduced water content within the crowns favors the accumulation of freezing tolerance during cold acclimation. Following 2 weeks at -2°C, no statistical differences in CMC between the two species were detected. In response to deacclimation at 4°C, both PR genotypes exhibited significant increases in CMC within 1 day at 4°C, with S16 having significantly higher CMC compared to T73, and is in agreement with the previous work in PR (Webster and Ebdon, 2005). The result suggested that S16 took up water more rapidly than T73 in response to temperature increases, which could negatively affect plant freezing tolerance as intracellular ice crystals are more likely to form once temperature drops below freezing.

Photosynthesis serves as the energy source for plants to accumulate freezing

tolerance at low temperatures (Andersson, 1944). The photochemical efficiency (Fv/Fm) represents the maximum quantum efficiency of photosystem II, indicating the capacity of excitation energy captured by photosynthesis apparatus. At low temperature, plants may adjust their photosystems to acclimate to current environmental conditions (Öquist et al., 1993). In our study, we observed a significant decrease in Fv/Fm in both genotype following 2°C acclimation (Fig. 4.3), which might be associated with the down regulation of photosynthetic related genes and the suppression of photosynthesis proteins production (Strand et al., 1997), or a feedback response induced by increased concentration of soluble sugars (Foyer et al., 1990). Among the genotypes, T73 exhibited significantly higher Fv/Fm compared to S16, indicating the photosynthesis apparatus of T73 was more active than S16 at 2°C. Following two weeks of -2°C acclimation (no light conditions), Fv/Fm slightly increased with no differences observed between the genotypes. As is described by Rizza et al. (2001), when plants were exposed to freezing temperature in the dark, the Fv/Fm might not be severely affected as the primary injury had been placed on the membranes. Since plants were fully exposed to darkness at -2°C, the photosynthesis apparatus may have already adjusted to the light conditions and the Fv/Fm increased as a result of homeostasis of photosystem II. Gray et al. (2003) also observed the recovery of Fv/Fm of cold acclimated plants in the dark.

In response to deacclimation at 4°C, both T73 and S16 exhibited a significant decrease in Fv/Fm following 1 day of deacclimation. The reason for the decrease in Fv/Fm might be that the photosynthesis apparatus were adjusting for the elevated temperature from -2°C to 4°C, and from the darkness to a light level of 150  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD. No differences were detected between the two genotypes at 4°C 1d. The Fv/Fm increased as deacclimation progressed, and the differences between the two

genotypes were observed at 4°C 3d, with T73 recovering Fv/Fm to a greater extent than S16. Similar response was found by Fracheboud et al.(1999) in *Zea mays* L. that cold tolerant species recovered faster than the sensitive species after low temperature exposure. Although the slower recovery might due to the chlorophyll deficiency in sensitive species as a result of oxidative stress response, further research is needed to investigate the relationship between photosynthetic apparatus and freezing tolerance in perennial ryegrass during deacclimation, such as chlorophyll content evaluation, gene expression investigation and photosynthetic protein quantification.

Quantum yield (Y) is a chlorophyll fluorescence parameter indicating the quantum efficiency of Photosystem II under steady light conditions, reflecting the current functioning of the photosynthesis apparatus. Quantum yield is also an important tool for plant stress conditions assessment, even though it is not used as extensively as Fv/Fm. Following 2°C acclimation, the Y in both genotypes decreased significantly, T73 had a statistically higher Y than S16, which is consistent with the Fv/Fm at 2°C (Fig. 4.3). The Y in S16 decreased to a greater extent than T73 in response to 2°C acclimation (T73: from 0.65 to 0.46, S16: from 0.67 to 0.43). During -2°C acclimation, the responses of Y in both genotype were similar to Fv/Fm that they both increased significantly. Given the fact that plants were under dark, the increases in Y might also due to the increased Photosystem II excitation capture capacity without photoinhibition. During deacclimation, the Y in both genotypes declined significantly, however, T73 had higher Y than S16. In general, the higher Y in T73 in response to temperature increases were associated with Fv/Fm during deacclimation. Similar results has been demonstrated by Dai et al. (2007) where a tolerant winter *Hordeum vulgare* L. cultivar exhibited significantly higher Y compared to the sensitive cultivar.

When the light energy absorbed by photosynthesis apparatus exceeds photochemical uses, excess light energy will either be reflected as fluorescence, or dissipated as heat. Non-photochemical quenching (NPQ) is a parameter that indicates the amount of heat dissipated, and is a protection system against the over-reduction of  $Q_A$  (Gilmore, 1997). In our study, during cold acclimation at 2°C, the NPQ in both T73 and S16 remained similar to that at 20°C. However, following -2°C acclimation, NPQ in both genotypes decreased significantly, but no differences between the two genotypes were detected. The decreased NPQ matched the increased Fv/Fm and Y, indicating more excitation energy flow into the Photosystem II. The down-regulation of NPQ at -2°C suggested the reduction of photoinhibition, as there were no light provided at -2°C. In response to deacclimation, at 4°C 1d, the NPQ of S16 exhibited a more rapid increase compare to T73. Given the fact that the yield of T73 was significantly higher than S16, the lower NPQ of T73 at this point compensated the rapid responses of quantum efficiency of electron transport. It also suggested that S16 was more susceptible to photoinhibition at this point. From 1d to 3d, the NPQ of T73 increased while S16 exhibited a significant decrease. The increase of NPQ in T73 might be a result of slower adaptation of quenching system to the new environment conditions, and the reduction in S16 might due to the damaged caused by higher light levels compared to the darkness at -2°C. However, further investigation on the responses of NPQ at early stages of deacclimation is necessary. Following 5 days of deacclimation at 4°C, there were no differences in NPQ system. Overall, the changes in NPQ during cold acclimation and deacclimation were related to the responses of Fv/Fm and Y, suggesting the photosynthesis apparatus of the two genotypes of perennial ryegrass is influenced under cold acclimation and deacclimation conditions.

Cold acclimation is associated with the gradual cessation of plant growth. For

instance, Gray et al. (1997) reported decreases in leaf expansion in winter rye during 5°C acclimation. Similarly, in our study, the leaf growth rate (LGR) decreased significantly in both T73 and S16 following 2°C acclimation, which is below 0.1cm (Fig. 4.4). Since plants were exposed to the same light levels compared to the non-acclimated treatment at 20°C (320 $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> PPFD), the decreased LGR is very likely induced by the decreased temperature (from 20° to 2°C), which had also been demonstrated by Gray et al. (1997). According to Savitch et al. (2002), the reduced growth of plants at low temperature might be associated with the inhibition of CO<sub>2</sub> assimilation, or the photodamages to photosystem II induced by high light levels. In addition, we compared the changes in leaf length within the 2 weeks of 2°C acclimation (1 d, 1 to 8 d, and 8 to 14 d). The data indicated both genotypes exhibited significantly higher LGR at the 1<sup>st</sup> day of 2°C than the rest, and S16 was significantly higher than T73. There were no differences detected between the LGR averaged over 1 to 8d and 8 to 15d. This informed us that during the early stage of 2°C acclimation, plants still maintained relatively high LGR, however, prolonged acclimation at 2°C resulted in reduced growth. In addition, the growth of T73 seemed to be less active than S16 during 1d of acclimation, indicating that the leaf growth of the tolerant genotype was more sensitive to temperature decrease. During -2°C acclimation in the dark, the LGR was close to zero for both genotypes, which was statistically lower than the growth rates observed at 2°C (Table 4.2).

Deacclimation induces a series of physiological and metabolic changes in plants that would stimulate the resumption of growth and also result in the losses in freezing tolerance (Sasaki et al., 2001; Arora et al., 2004; Kalberer et al., 2006; Huang et al., 2014). The ability to re-acclimate is significantly diminished once plants resume growth (Leinonen et al., 1997; Rapacz , 2002), although this is also influence



by light level and photoperiod (, 2002). Our data revealed that at 4°C 1d, the LGR in both genotypes increased slightly, however was still not significantly different from LGR at -2°C (Fig. 4.4). Plants remained the same levels of LGR in both genotypes in response to 5 days of 4°C deacclimation. This indicated that a short-time deacclimation at 4°C and under 150  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD was not enough to induce a significant amount of regrowth in plants, although a trend was evident for increases in LGR. This indicates that high temperatures and differences in the light levels could lead to enhanced growth and therefore reduced re-acclimation in response to freezing temperatures.

Unlike leaf growth, the relative tiller number (TN) served as a measure of the development or senescence of leaf tissues in response to cold acclimation and deacclimation. The means of TN averaged over T73 and S16 during 2°C acclimation was significantly higher than the -2°C means (2°C: 100.9%, -2°C: 92.93%, LSD=7.1). Given the fact that 2°C resulted in significant decreases in LGR, it is possible that plants had allocated the energy for leaf growth to the build up of freezing tolerance during 2°C acclimation rather than development of new plant tissues. In response to deacclimation, by contrasting the means at 4°C 1d to 4°C 5d, the results showed that plants exposed to 4°C 5d had significantly higher TN than at 4°C 1d, revealing that plants were already adapted to temperature increases and were ready for new tiller growth.

In summary, T73 was found to have higher freezing tolerance than S16 following cold acclimation and deacclimation. The photosystem II efficiency of T73 was significantly higher than S16 following 2°C acclimation, as shown by higher Fv/Fm and Y. In response to deacclimation, T73 also exhibited higher Y, but lower NPQ. The changes in chlorophyll fluorescence parameters suggesting that the

photosystem apparatus of T73 was more responsive to temperature changes than S16, thus may have prevented the damages to the photosynthesis machinery brought by low temperature. Higher CMC of S16 following 2°C acclimation and 4°C 1d deacclimation indicated higher water content within the crowns of S16, which induced higher susceptibility to low temperature injury than T73. Therefore, decreasing turf water content during freezing temperatures is critical for winter survival. On golf courses, sand is more preferable than soil due to their poorer ability to hold water, therefore reducing the crown moisture content during winter months thus makes the plants more tolerant to low temperature injuries. Using impermeable covers are also important, as they are able to prevent crown hydration and serve as an insulation to maintain plant winter hardiness (Skorulski, 2002). In addition, shaded areas on golf courses need to be eliminated, as plants under shade are more susceptible to low temperature injury than plants under sun, which is associated with higher crown moisture content and less ability to photosynthesize and accumulate protective compounds (Skorulski, 2002). Low temperature inhibited both tiller and leaf growth, however once temperature increases, the tiller growth resumes faster than leaf growth, as significantly higher TN was observed at 4°C 5d compared to 4°C 1d, however LGR was not significantly influenced by temperature increase. According to Webster and Ebdon (2005), nitrogen and potassium play a role in regulating plant winter hardiness, as they were able to affect plant crown moisture content and stimulate shoot growth. Therefore, from management perspective, avoiding high rates of soluble nitrogen as an early fall application is critical for enabling plant survival through winter. In addition, applying potassium would contribute to the building up of freezing tolerance, as it was previously reported to interact with nitrogen and affect shoot growth, carbohydrate reserves and hydration levels thus affect freezing

tolerance (Monroe et al., 1969; Christian et al., 1981; Ebdon et al., 1999; Webster and Ebdon, 2005). For future research on cold acclimation and deacclimation mechanisms in PR, more detailed investigations from genetic to whole plant levels is necessary. We suggested to provide research on the gene expression in response to temperature changes such as the cold regulated genes, the photosynthesis genes and carbohydrate synthesis genes. In addition, changes in metabolic responses such as proteins and carbohydrates are also needed to provide more information for genetic analysis.

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Figure 4.1. Changes in freezing tolerance ( $LT_{50}$ ) of T73 and S16 following cold acclimation (2,  $-2^{\circ}\text{C}$ ) and deacclimation ( $4^{\circ}\text{C}$  1d, 5d). Vertical bars are LSD values ( $P \leq 0.05$ ) indicating significant differences between the two genotypes among different temperature treatments.

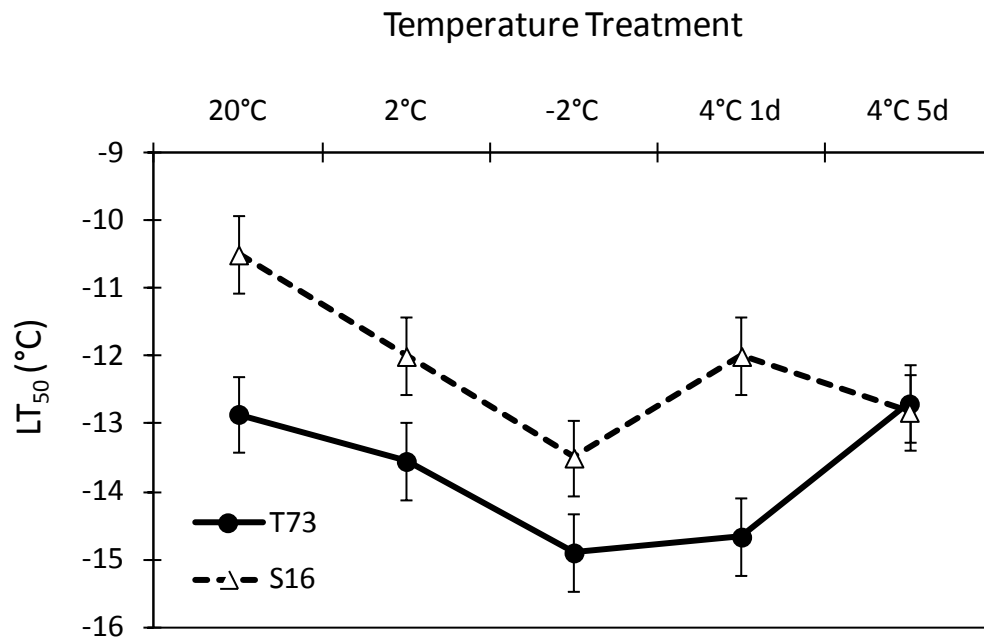


Figure 4.2. Changes in crown moisture content (CMC) of T73 and S16 following cold acclimation (2, -2°C) and deacclimation (4°C 1d, 5d). Vertical bars are LSD values ( $P \leq 0.05$ ) indicating significant differences between the two genotypes among different temperature treatments.

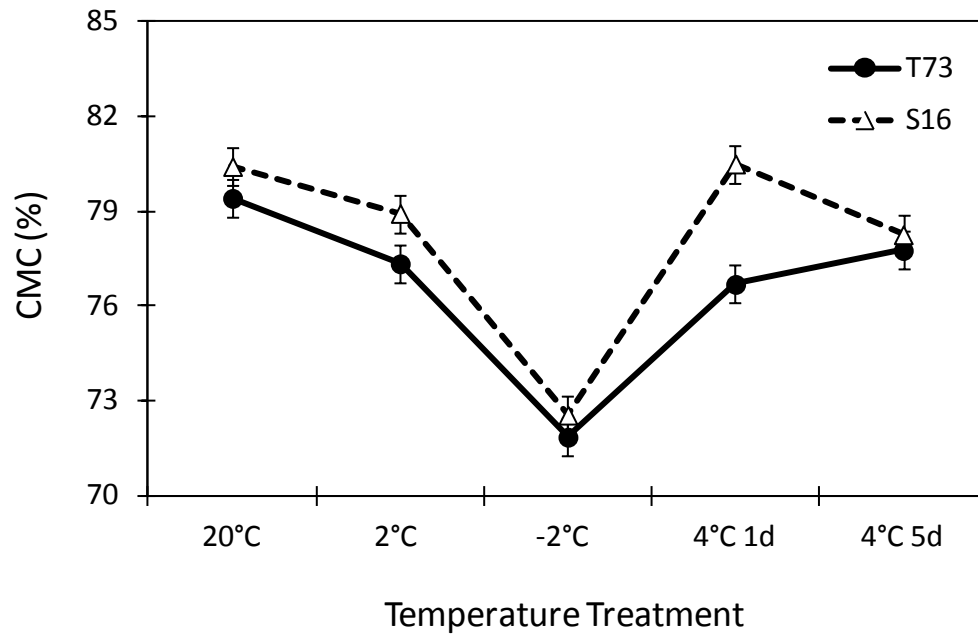


Figure 4.3. Changes in photochemical efficiency ( $F_v/F_m$ ), quantum yield ( $Y$ ), and non-photochemical quenching (NPQ) of T73 and S16 following cold acclimation (2, -2°C) and deacclimation (4°C 1d, 5d). Vertical bars are LSD values ( $P \leq 0.05$ ) indicating significant differences between the two genotypes among different temperature treatments.

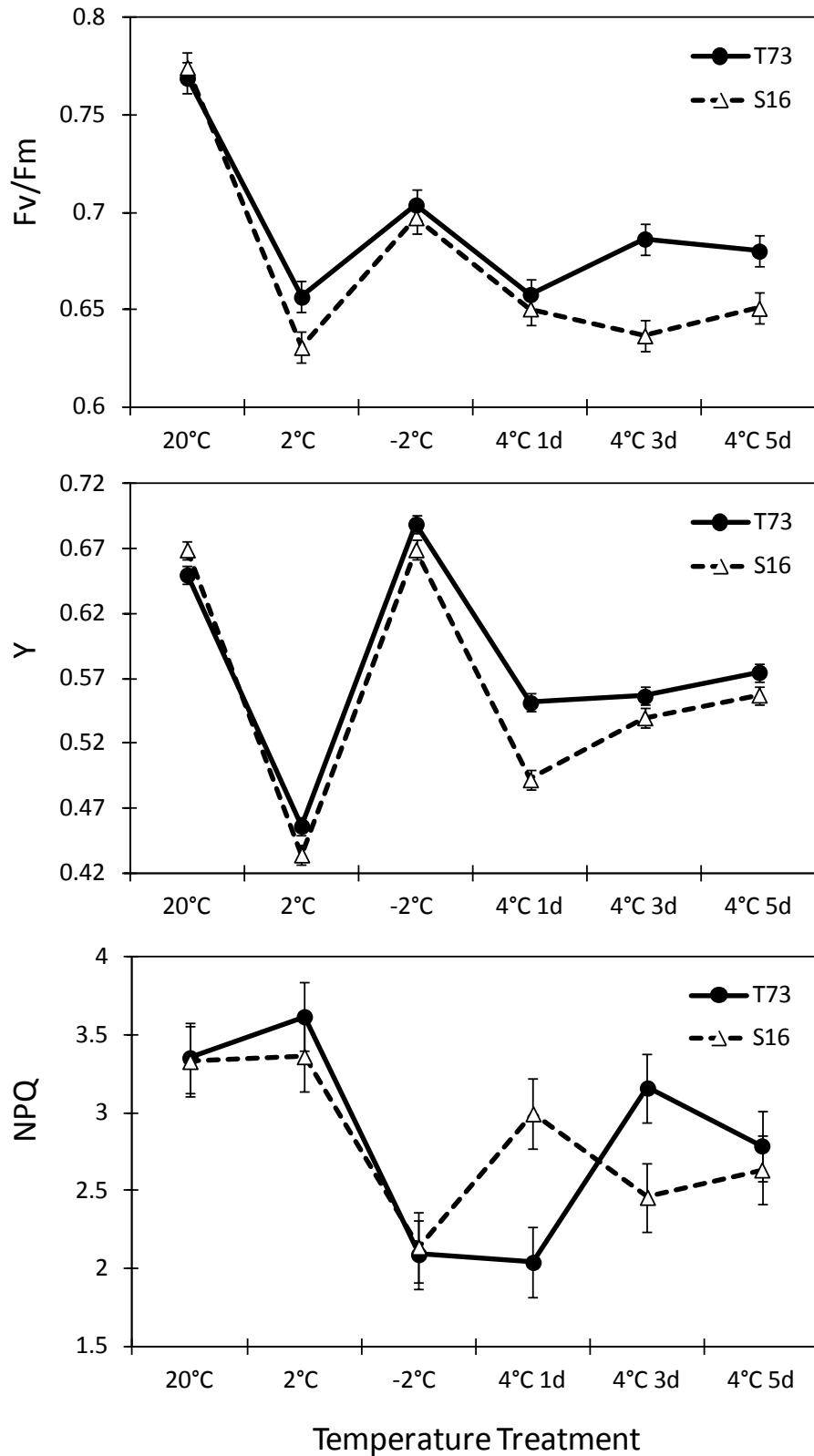


Figure 4.4. Changes in leaf growth rate (LGR) of T73 and S16 following cold acclimation (2, -2°C) and deacclimation (4°C 1d, 5d). Vertical bars are LSD values ( $P \leq 0.05$ ) indicating significant differences between the two genotypes among different temperature treatments.

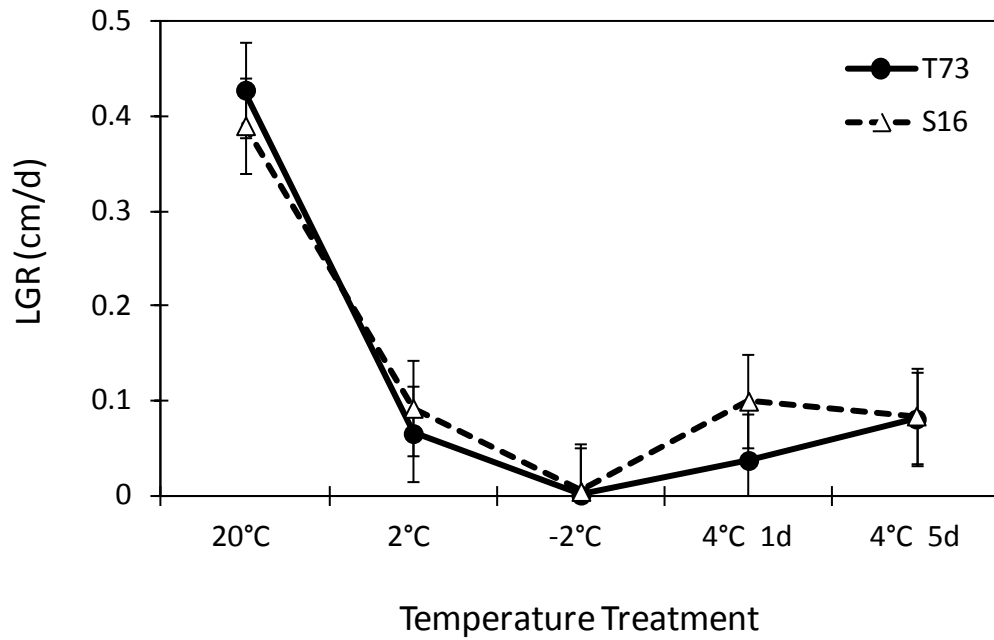


Figure 4.5. Changes in tiller number (TN) of T73 and S16 following cold acclimation (2, -2°C) and deacclimation (4°C 1d, 5d). Vertical bars are LSD values ( $P \leq 0.05$ ) indicating significant differences between the two genotypes among different temperature treatments.

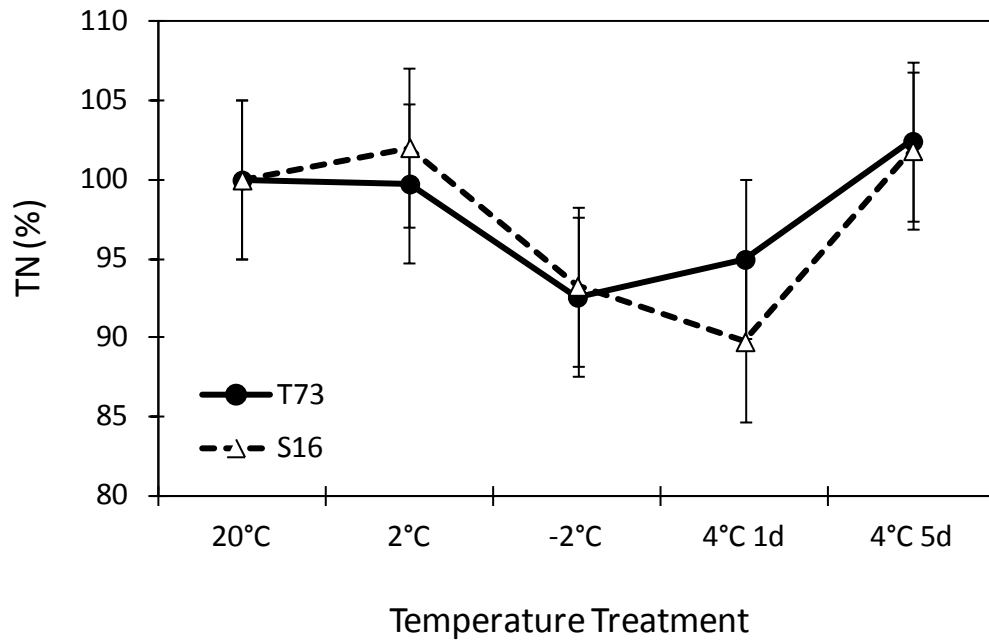


Table 4.1. Means from ANOVA of freezing tolerance ( $LT_{50}$ ) of four freezing tolerant PR genotypes (T73, T74, T88, T89) and four freezing sensitive PR genotypes (S08, S16, S23, S38) following non-acclimation (20°C for 2 weeks), cold acclimation (2 and -2°C for 2 weeks) and deacclimation (8°C 3d) in Experiment 1.

Genotypes	$LT_{50}$ (°C)				
	Overall	20°C	2°C	-2°C	8°C 3d
T73	-13.1 c <sup>†</sup>	-9.3 a	-11.7 a	-21.9 d	-9.0 a
T74	-12.5 abc	-9.3 a	-12.1 a	-19.2 ab	-9.5 a
T88	-12.7 bc	-9.9 a	-11.3 a	-20.7 cd	-9.3 a
T89	-12.7 bc	-9.4 a	-11.4 a	-20.3 bc	-9.4 a
S08	-12.1 ab	-9.3 a	-11.9 a	-18.1 a	-9.3 a
S16	-11.9 a	-9.2 a	-11.5 a	-17.9 a	-9.1 a
S23	-12.5 abc	-9.5 a	-11.3 a	-19.9 bc	-9.3 a
S38	-13.1 c	-9.4 a	-11.5 a	-21.8 d	-9.4 a

<sup>†</sup>Means followed by the same letter within each column for each genotype are not significantly different based on Fisher's protected LSD ( $P \leq 0.05$ )

Table 4.2. Results and contrasts from ANOVA of freezing tolerance (LT<sub>50</sub>), crown hydration (CH), leaf growth rate (LGR), tiller growth rate (TGR) as influenced by genotype (T73, S16) in response to non acclimation (20°C for 2 wk), cold acclimation (2°C for 2 wk, -2°C for 2 wk), and deacclimation (4°C 1d, 5d) treatments.

Source of variation	df	LT <sub>50</sub> ----- °C -----	CH ----- % -----	LGR ----- cm -----	TN ----- % -----
Genotype	1	***	***	NS	NS
Environment	4	***	***	***	*
Control vs. All	1	***	***	***	NS
AC vs. DAC	1	**	***	NS	NS
Within AC	1	***	***	*	*
Within DAC	1	NS	NS	NS	**
Species x Environment	4	***	**	NS	NS
(T73 vs. S16) x (Control vs. All)	1	NS	NS	NS	NS
(T73 vs. S16) x (AC vs. DAC)	1	NS	NS	NS	NS
(T73 vs. S16) x (Within AC)	1	NS	NS	NS	NS
(T73 vs. S16) x (Within DAC)	1	**	***	NS	NS

NS, Nonsignificant

\*\*\*, \*\*, \* Significant at P ≤ 0.001, 0.01, and 0.05 probability.

Table 4.3. Results and contrasts from ANOVA of photochemical efficiency (Fv/Fm), quantum yield (Y), and non-photochemical quenching (NPQ) as influenced by genotype (T73, S16) in response to non acclimation (20°C for 2 wk), cold acclimation (2°C for 2 wk, -2°C for 2 wk), and deacclimation (4°C 1d, 3d, 5d) treatments.

Source of variation	df	Fv/Fm	Y	NPQ
Genotype	1	***	***	NS
Environment	5			
Control vs. All	1	***	***	***
AC vs. DAC	1	**	***	NS
Within AC	1	***	***	***
Within DAC	2	NS	***	NS
Species x Environment	5			
(T73 vs. S16) x (Control vs. All)	1	**	***	NS
(T73 vs. S16) x (AC vs. DAC)	1	NS	NS	NS
(T73 vs. S16) x (Within AC)	1	NS	NS	NS
(T73 vs. S16) x (Within DAC)	2	**	***	***

NS, Nonsignificant

\*\*\*, \*\*, \* Significant at  $P \leq 0.001$ , 0.01, and 0.05 probability.



## CHAPTER 5

### CONCLUSION

Although winter injury of cool-season grasses can be caused by multiple factors, the ability of plants to maintain high freezing tolerance throughout winter months is critical for winter survival. To date, most research has focused on the factors influencing cold acclimation capacity of plants, whereas much less is known about mechanisms underlying losses of freezing tolerance that can occur in response to warming events from winter through spring months. Therefore, the overall goal of this research was to examine mechanisms responsible for freezing tolerance differences among cool-season turfgrasses, with a focus on cold acclimation capacity and deacclimation resistance. To address this goal, we conducted three controlled-environment studies to better understand the physiological mechanisms of freezing tolerance using grasses that differed in cold acclimation and deacclimation.

Our results suggested that the up-regulation of carbon metabolism was triggered very early in the deacclimation response (ie. 1 d), as measured by chlorophyll fluorescence, photosynthesis, and respiration rates, which coincided with the losses of freezing tolerance for annual bluegrass and creeping bentgrass. Moreover, we observed significant changes in hormone contents of leaves and crowns of annual bluegrass and creeping bentgrass, such as abscisic acid, auxin, salicylic acid, and jasmonic acid, which could contribute to differences in deacclimation resistance and overwintering capacity. Therefore, practical application of plant growth regulators or other compounds that may influence endogenous hormones may help to mitigate freezing tolerance losses and protect the grasses from winter injury.

Using perennial ryegrass, we confirmed sensitivity of carbon metabolism to short-term warm temperature exposures, which was also accompanied by increased

crown moisture content. These physiological changes may aid in a faster recovery and regrowth during late winter and early spring. However, in response to mid-winter thaw events, these physiological changes could make the plant more susceptible to low temperature kill when plants are then re-exposed to freezing temperatures. Therefore, minimizing mid-winter fluctuation, inhibit early up-regulation of carbon metabolism, and reduce crown moisture content are necessary for maintaining plants winter hardiness. On golf courses, a covering system (i.e. impermeable cover) is suggested to be used during winter months as it would prevent turf from hydration and insulate them from extensive temperature fluctuations (Skorulski, 2002). In addition, reducing the fertilizer application in early fall can also reduce crown moisture content and maintains plant winter hardiness (Webster and Ebdon, 2005).

In the future, a more detailed focus on deacclimation mechanisms is warranted, particularly greater information on gene expression changes in response to freezing and freeze-thaw cycles such as the cold regulated genes, the photosynthesis genes and carbohydrate synthesis genes are encouraged. In addition, physiological and metabolic changes such as proteins and carbohydrates are also needed to provide more information for genetic analysis.

## APPENDIX

### FREEZE TEST PROGRAM

Freeze tests were conducted in a programmable freeze chamber (Tenney TC Series Cycling Test Chamber, SPX Thermal Product Solutions, White Deer, PA). The freeze tests were programmed as follows (using the program for 2°C acclimation treatment as an example):

Step 1. Auto start program at 3:00 am on 04/03/14 at 2°C

Step 2. Ramp 4 hours to -6°C

Step 3. Soak at -6°C for 1 hour

Step 4. Ramp 1.5 hours to -9°C

Step 5. Soak at -9°C for 1 hour

Step 6. Ramp 1.5 hours to -12°C

Step 7. Soak at -12°C for 1 hour

Step 8. Ramp 1.5 hours to -15°C

Step 9. Soak at -15°C for 1 hour

Step 10. Ramp 1.5 hours to -18°C

Step 11. Soak at -18°C for 1 hour

Step 12. Ramp 11 hours to 4°C

Step 13. End step, hold.

Temperature in the freeze chamber were set to decrease at the rate at 2°C per hour. For the 20°C non-acclimation treatments, the temperature start point in Step 1 was set at 4°C. For cold acclimation treatments at 2 and -2°C, the start points were set at 2 and -2°C, respectively. For deacclimation treatments at 8°C and 4°C, the start points were set at 8 and 4°C, respectively.

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